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October 26, 2007

Mail Stop Appeal Brief – Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Applicant: George Blanck et al.
Serial No.: 10/711,101
Filing Date: 08/23/2004
For: OCT-1 as an Oncoprotein
Our Reference: 1372.183.PRC

Examiner: Tracy Ann Vivlemore
Art Unit: 1635
Confirmation No.: 5100

Dear Sir/Madam:

Enclosed please find the following:

1. Brief of Appellant having a Certificate of Mailing dated June 13, 2007;
2. *Opalinska et al.* Nature Reviews Drug Discovery, 2001, vol. 1, pg. 503-514;
3. U.S. Patent No. 5,776,502 to *Foulkes et al.*;
4. U.S. Patent No. 6,136,779 to *Foulkes et al.*;
5. Unknown author, Cell line Indexes, http://www.atcc.org/document/pdf/CellCatalog.TumorLines.pdf
6. Müthing et al., *Preferential Binding of the Anticancer Drug rViscumin (Recombinant Mistletoe Lectin) to Terminally α2-6-sialylated Neolacto-Series Gangliosides*; and
7. Self-addressed, postage prepaid post card to serve as a receipt for items 1 and 6.

Very respectfully,

SMITH & HOPEN

By: Thomas E. Toner
tom.toner@smithhopen.com

TET/at
Enclosures

CERTIFICATE OF MAILING
(37 C.F.R. 1.10)

I HEREBY CERTIFY that this Appeal Brief is being deposited with the United States Postal Service as "Express Mail Post Office to Addressee" Mailing Label No. EM036786938US, addressed to: Mail Stop Appeal Brief - Patents, Commissioner for Patents, P.O. Box 1450 Alexandria, VA 22313-1450 on. October 26, 2007.

Date: **October 26, 2007**

April Turley
April Turley

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BOARD OF PATENT APPEALS AND INTERFERENCES

Application No.	: 10/711,101	Confirmation No.: 5100
Applicants	: George Blanck	
	: Kimberly Palubin	
	: Aaron Osborne	
Filed	: 08/23/2004	
Examiner	: Tracy Ann Vivlemore	
Docket No.	: 1372.183.PRC	
Customer No.	: 21,901	
For	: OCT-1 as an Oncoprotein	

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313

BRIEF OF APPELANT

Sir/Madam:

In furtherance of its appeal from the Final Rejection mailed December 13, 2006, Applicant hereby submits its Appeal brief.

TABLE OF CONTENTS

1. REAL PARTY IN INTEREST

The real party in interest is the University of South Florida, the assignee of record, which is a state university, organized under the laws of the State of Florida, as evidenced by the assignment set forth at Reel 015310, Frame 0262.

2. RELATED APPEALS AND INTERFERENCES

None

3. <u>STATUS OF CLAIMS</u>

Canceled claims:	1-3
Withdrawn Claims:	4-8
Rejected claims:	9-13
Claims under appeal:	9-13

4. STATUS OF AMENDMENTS

No amendments have been made subsequent to the final rejection by the Office.

5. SUMMARY OF CLAIMED SUBJECT MATTER

Citations to the specification are by page and line number. A concise explanation of the invention defined in the claims involved in this appeal is provided below. Claim 9 is the only independent claim on appeal.

Claim 9 recites a method for treating a tumor (Page 7, lines 1-3) in a subject in need thereof, comprising administering to said subject a therapeutically effective amount of an Oct-1 inhibitor (Page 7, lines 1-3; page 12, line 22 – Page 13, line 6).

6. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

I. Whether the Office erred in rejecting claims 9 through 13 under 35 U.S.C. 112, first paragraph as failing to comply with the written description requirement alleging the specification fails to describe any inhibitors of OCT-1 that treat tumors in a subject.

II. Whether the Office erred in rejecting claims 9 through 13 under 35 U.S.C. 112, first paragraph as failing to comply with the enablement requirement based on the fact that the delivery of nucleic acid inhibitors is allegedly recognized in the art as unpredictable.

7. ARGUMENT

I. The Office erred in rejecting claims 9 through 13 under 35 U.S.C. 112, first paragraph as failing to comply with the written description requirement.

The written description requirement of 35 U.S.C. § 112 [¶1] is separate from the enablement requirement. Vas-Cath Inc. v. Mahurkar, 935 F.2d 1555, 1563, 19 USPQ2d 1111, 1116-17 (Fed. Cir. 1991). Adequate written description must be determined from the disclosure considered as a whole. Reiffin v. Microsoft Corp., 214 F.3d 1342, 1346, 54 USPQ2d 1915, 1917 (Fed. Cir. 2000). To fulfill the written description requirement, a patent specification must describe an invention in sufficient detail that one skilled in the art can clearly conclude that the inventor invented the subject matter claimed. Regents of the Univ. of Cal. v. Eli Lilly & Co., 119 F.3d 1559, 1566, 43 USPQ2d 1398, 1404 (Fed. Cir. 1997); Lockwood v. American Airlines, 107 F.3d 1565, 1572, 41 USPQ2d 1961, 1966 (Fed. Cir. 1997). The knowledge of one skilled in the art must be considered. Bilstad v. Wakalopulos, 386 F.3d 1116, 1126, 72 USPQ2d 1785, 1792 (Fed. Cir. 2004).

Here, the claims require administering an Oct-1 inhibitor to a subject. According to the examiner, however:

> While one might be able to identify inhibitors of Oct-1, the structure and function of Oct-1 will not lead the skilled artisan to envision compounds having the function recited by the claims, of treating a tumor *in vivo.* The claimed invention encompasses the use of inhibitors of Oct-1 of any type, including nucleic acids, antibodies and small molecules but the specification describes only a single antisense vector capable of inhibiting Oct-1 *in vitro*. Neither the specification nor the prior art describe any inhibitors of Oct-1 that treats tumors in a subject. (Office Action, December 13, 2006, Page 3 and 4).

The examiner does not dispute that the specification discloses an agent capable of inhibiting Oct-1, nor does the examiner dispute the existence of numerous other Oct-1 inhibitors. The examiner, however, argues that although "although some inhibitors of Oct-1 are known from the prior art" that "these known inhibitors are not describe by the prior art as able to treat tumors in a subject." (*Id.* at page 4).

The absence of a working example is not fatal to compliance with the written description requirement where, as here, the specification otherwise conveys to one skilled in the art that the applicant's possessed the claimed invention at the time of filing. See In re Wands, 858 F.2d 731, 736, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). (Whether a working example is present is a factor that may be considered in determining whether undue experimentation is required to practice the invention).

A specification may, within the meaning of 35 U.S.C. § 112, ¶ 1 contain a written description of a broadly claimed invention without describing all the species that claim encompasses. Utter v. Hiraga, 845 F.2d 993 (Fed.Cir.1988). Thus, the mere fact that the specification fails to describe the full genus of encompassed compounds that have the function of inhibiting OCT-1 does not mean that the application fails to meet the written description requirement. Genus claims are sufficiently enabled so long as one of ordinary skill in the art can "visualize or recognize the identity of the members of the genus" from reading the specification. Regents of the Univ. of Cal. v. Eli Lilly & Co., 119 F.3d 1559, 1568 (Fed.Cir.1997).

The examiner has not disputed the veracity of the results presented in the specification or explained why such a disclosure would not have conveyed to one skilled in the art that patentees were in possession of the claimed invention. If it is the examiner's position that undue experimentation would have been required to determine the appropriate procedures for administering an Oct-1 inhibitor *in vivo*, then the proper rejection is failure to provide an enabling disclosure; which is addressed below.

II. The Office erred in rejecting claims 9 through 13 under 35 U.S.C. 112, first paragraph as failing to comply with the enablement requirement.

Enablement is a legal determination of whether a patent enables one skilled in the art to make and use the claimed invention (Raytheon Co. v. Roper Corp., 724 F.2d 951, 960, 220 USPQ 592, 599 (Fed.Cir.1983)), and is not precluded even if some experimentation is necessary. All that is required is the amount of experimentation needed must not be unduly extensive. (Atlas Powder Co. v. E.I. Du Pont De Nemours & Co., 750 F.2d 1569, 1576, 224 USPQ 409, 413 (Fed.Cir.1984)). Furthermore, a patent need not teach, and preferably omits, what is well known in the art.

The examiner bears the initial burden of showing that a claimed method is not enabled. See In re Wright, 999 F.2d 1557, 1561-62, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993) ("[T]he PTO bears an initial burden of setting forth a reasonable explanation as to why it believes that the scope of protection provided by that claim is not adequately enabled by the description of the invention provided in the specification of the application."). The invention that must be enabled to satisfy § 112 is the invention defined by the claims. See CFMT, Inc. v. Yieldup Int'l Corp., 349 F.3d 1333, 1338, 68 USPQ2d 1940, 1944 (Fed. Cir. 2003) ("Title 35 does not require that a patent disclosure enable one of ordinary skill in the art to make and use a perfected, commercially viable embodiment absent a claim limitation to that effect.").

The enablement of a genus under § 112, ¶ 1, can be established by showing the enablement of a representative number of species within the genus. (Regents of the Univ. of Cal. 119 F.3d 1559, 1568 (Fed.Cir.1997)). Applicants "are not required to disclose every species encompassed by their claims even in an unpredictable art." See In re Angstadt, 537 F.2d at 502-03, 190 USPQ at 218 (deciding that applicants "are not required to disclose every species encompassed by their claims even in an unpredictable art" and that the disclosure of forty working examples sufficiently described subject matter of claims directed to a generic process). Disclosures of working examples that sufficiently describe the subject matter of claims are sufficient to support claims directed to a generic process. (*Id.*). Moreover, naming representative compounds encompassed by generic claim language is clearly not required by §112 or any other provision of the statute. In re Robins, 429 F.2d 452, 456-57, 166 USPQ 552, 555 (Cust. & Pat.App.1970). However, mention of representative compounds may provide an implicit description upon which to base generic claim language, even where no explicit description of a generic invention is to be found in the specification. (*Id.*)

Moreover, the Patent and Trademark Office has promulgated Guidelines to be used by patent examiners in determining whether patent applications meet the written description requirement. According to the Guidelines, an application drawn to a genus meets the written description requirement if it either:

> (1) describes "a representative number of species by actual reduction to practice;" or
> (2) discloses "relevant, identifying characteristics, i.e. structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying characteristics, sufficient to show the applicant was in

> possession of the claimed genus." (Guidelines for Examination of Patent Applications Under the 35 U.S.C. § 112.1 "Written Description Requirement", 66 Fed.Reg. 1099, 1106 (Jan. 5, 2001) (hereinafter "Guidelines ")).

Here, Applicant has set forth precise detail about the structural and chemical properties of the Oct-1 mechanism. Inhibitors of Oct-1 are similarly detailed by their functional characteristics coupled with the known correlation between Oct-1 function and structure. The Examiner's Action itself points out that numerous inhibitors of Oct-1 are known. The very references cited by the Examiner show that Oct-1 inhibiting substances are sufficiently well known in the art such that one could routinely apply Applicant's techniques to inhibit Oct-1 without undue experimentation. Accordingly, the working example provided in the specification coupled with the knowledge of the prior art is sufficient to enable the claimed genus of compounds. Enablement does not require that the specification disclose that which is well known in the art. Hybritech Inc. v. Monoclonal Antibodies, Inc., 802 F.2d at 1384, 231 USPQ at 94.

It is well settled that all the disclosures in a reference must be evaluated and that a reference is not limited to the disclosure of specific working examples. In re Mills, 470 F.2d 649, 651, 176 USPQ 196, 198 (CCPA 1972). For example, the specification provides detailed disclosure regarding the construction of a vector comprising antisense Oct-1 cDNA which can be used to inhibit Oct-1 expression. The specification continues to discuss screening assays which can be used for identifying such inhibitors. Such screening assays, once identified, can be routinely used by those of ordinary skill in the art to screen compounds for similar activity. The Office has advanced no specific reasoning as to why the compound screening assays outlined in the specification would be unsuccessful in identifying other similar agents, which can act as inhibitors of Oct-1 and therefore has not provided sufficient evidence to support a finding of lack of enablement of the claimed invention.

Methods of modulating gene expression, both in vitro and in vivo, were well known prior to Applicant's filing date. For example, U.S. Patent Nos. 5,776,502[1] and 6,136,779[2] teach a method of modulating, in vivo, the expression of a gene of interest, transcriptionally, which

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[1] U.S. Patent No. 5,776,502 to *Foulkes et al.*, Filed June. 2, 1995
[2] U.S. Patent No. 6,136,779 to *Foulkes at al.*, Filed Jan. 6, 1997

encodes a protein where the expression of which is associated with a defined physiological or pathological effect.

Moreover, methods to identify the necessary characteristics, including affinity, of expression modulators, such as those used in the invention were known in the art at the time the application was filed. Furthermore, the Office has not produced any evidence that undue experimentation would be required by those skilled in the art to practice this invention. Rather, the Office has cited *per se* rules, which have been previously and expressly condemned by the courts, relying on generalized articles. With regard to the paragraph cited by the Office, *Opalinska* expressly states that "*as a general rule*, oligonucleotides are taken up primarily through a combination of adsorptive and fluid-phase endocytosis."[3] Whether the specification for a challenged claim meets the enablement requirement is a question of fact to be assessed on a case-by-case basis. Vas-Cath Inc. v. Mahurkar, 935 F.2d 1555, 1561, 1563 (Fed.Cir.1991); Eli Lilly, 119 F.3d at 1566. The specification of the application and the references highlighted by the Office show that the modulation of Oct-1 was known in the art. Here the Office has not provided any analysis specific to the claims to show that one skilled in the art would not be able to practice this invention, as is its burden.

The specification also shows that the 5637 cell line correlates to the breadth of the claims. The issue of "correlation" is dependent on the state of the prior art, and the art of record is such that this particular model is recognized as correlating to tumor growth. For example, The ATCC CULTURES™ cell line list,[4] attached hereto, lists tumor cell lines from a variety of species. Cell lines that are known to be from metastatic sites, including the 5637[5] line used as a prophetic example, are listed. See also *Muthing,* wherein the 5637 cell line was selected as representing malignant cells of epithelial morphology. (*Preferential Binding Of The Anticancer Drug Viscumin (Recombinant Mistletoe Lectin) To Terminally α2-6-Sialylated Neolacto-Series Gangliosides*; Muthing *et al.*, Glycobiology, Vol. 12, no. 8, pp 485-497, 2002).

Applicant's specification clearly shows the requisite correlation of the prophetic example to the claimed activity since the 5637 cell line possess "increased Oct-1 binding activity" and that "Oct-1 is hypophosphorylated in 5637 cells, which subsequently increases its

[3] *Opalinksa et al.* Nature Reviews Drug Discovery, 2002, vol. 1, p.503-514, page 511, col. 2.
[4] http://www.atcc.org/documents/pdf/CellCatalog/TumorLines.pdf
[5] *Id.*, Page 142

DNA binding activity" and have a "high level of active Oct-1 as compared to non-cancerous cells." (Paragraph 31).

As such, the prophetic example should be accepted as correlating since the Office has not offered any evidence that this model does not correlate to *in vivo* activity. Even with such evidence, the Office must weigh the evidence for and against correlation to show whether one skilled in the art would accept the model as reasonably correlating to the condition. In re Brana, 51 F.3d 1560, 1566, 34 USPQ2d 1436, 1441 (Fed. Cir. 1995) (reversing the PTO decision based on finding that *in vitro* data did not support *in vivo* applications).

The Office has not met the initial burden to give relevant reasons for the lack of enablement. Similarly the Office has not given case-specific reasons for a conclusion of lack of correlation of an *in vitro* example. Applicant is not required to establish a rigorous or an invariable exact correlation. (Cross v. Iizuka, 753 F.2d 1040, 1050, 224 USPQ 739, 747 (Fed. Cir. 1985)). Based upon the relevant evidence as a whole, there is a reasonable correlation between the disclosed *in vitro* utility and an *in vivo* activity, and therefore a rigorous correlation is not necessary here; therefore, *in vivo* activity is reasonable based upon the probative evidence.

Lastly, the claims are directed to a "method of treating a tumor in a subject in need thereof, comprising administering to said subject a therapeutically effective amount of an Oct-1 inhibitor." (claim 9). Thus, while it is fair to say that the claims encompass a method that achieves a clinically effective therapeutic response, they do not require it; the rejection being based on the mode and the concentration. Cf. In re Cortright, 165 F.3d 1353, 49 USPQ2d 1464 (Fed. Cir. 1999) (claims to a method of "treating scalp baldness" could be enabled even if the method did not produce a full head of hair). Moreover, a claim may encompass inoperative embodiments and still meet the enablement requirement of 35 U.S.C. § 112, first paragraph. See Atlas Powder Co. v. E.I. Du Pont De Nemours & Co., 750 F.2d 1569, 1576, 224 USPQ 409, 413 (Fed. Cir. 1984), In re Angstadt, 537 F.2d 498, 504, 190 USPQ 214, 218 (CCPA 1976), In re Cook, 439 F.2d 730, 732, 169 USPQ 298, 300 (CCPA 1971). And the stage at which an invention in this field become useful is well before it is ready to be administered to humans." In re Brana, 51 F.3d 1560, 1568, 34 USPQ2d 1436, 1442 (Fed. Cir. 1995). (While the Brana court referred to "usefulness", the rejection on appeal was for nonenablement. See id. at 1564, 34 USPQ2d at 1439.)

Here the examiner does not contend that the term "therapeutically effective amount," which is the only limitation which indicates a clinically therapeutic response, is not enabled. The potential problems identified by the examiner may indeed complicate the use of nucleic acid inhibitors in a subject, but such problems need not be overcome in order to administer to said subject an Oct-1 inhibitor - all that is required by the claims. Thus, the examiner has not adequately explained why practicing the claimed method would have required undue experimentation.

8. CLAIMS APPENDIX

Serial No: 10/711,101
Filed: 23 August 2004
Title: Oct-1 as an Oncoprotein

REJECTED CLAIMS

9. A method for treating a tumor in a subject in need thereof, comprising administering to said subject a therapeutically effective amount of an Oct-1 inhibitor.

10. The method of claim 9 wherein an Oct-1 inhibitor inhibits Oct-1binding activity.

11. The method of claim 9 wherein an Oct-1 inhibitor inhibits Oct-1 mRNA function.

12. The method of claim 9 wherein the Oct-1 inhibitor is a vector containing an Oct-1 antisense sequence.

13. The method of claim 9 wherein the Oct-1 inhibitor is an RNA inhibitor molecule.

<u>9. EVIDENCE APPENDIX</u>

1. Opalinksa et al., *Nucleic-Acid Therapeutics: Basic Principles and Recent Applications*, Nature Reviews Drug Discovery, 2002, vol. 1, p.503-514

2. U.S. Patent No. 5,776,502 to *Foulkes et al.,* Filed June. 2, 1995

3. U.S. Patent No. 6,136,779 to *Foulkes at al.*, Filed Jan. 6, 1997

4. Unknown author, *Cell line Indexes*, http://www.atcc.org/documents/pdf /CellCatalog/TumorLines.pdf (last accessed Wednesday, October 10, 2007).

5. Müthing et al., *Preferential Binding of the Anticancer Drug rViscumin (Recombinant Mistletoe Lectin) to Terminally α2-6-sialylated Neolacto-Series Gangliosides*, Glycobiology, 2002, vol. 12, p.485-497

10. RELATED PROCEEDINGS APPENDIX

None

CONCLUSION

Applicant respectfully submits that the rejections of claim 9-13 under 35 U.S.C. §112, first paragraph are improper and should be withdrawn. Fairness to Applicant requires reversal of the final rejection; therefore, such reversal is solicited.

Very respectfully,

SMITH & HOPEN, P.A.

By:____________________

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Dated: October 26, 2007

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April Turley

NUCLEIC-ACID THERAPEUTICS: BASIC PRINCIPLES AND RECENT APPLICATIONS

Joanna B. Opalinska and Alan M. Gewirtz‡*

The sequencing of the human genome and the elucidation of many molecular pathways that are important in disease have provided unprecedented opportunities for the development of new therapeutics. The types of molecule in development are increasingly varied, and include antisense oligonucleotides and ribozymes. Antisense technology and catalytic nucleic-acid enzymes are important tools for blocking the expression of abnormal genes. One FDA-approved antisense drug is already in the clinic for the treatment of cytomegalovirus retinitis, and other nucleic-acid therapies are undergoing clinical trials. This article reviews different strategies for modulating gene expression, and discusses the successes and problems that are associated with this type of therapy.

EXOGENOUS NUCLEIC ACIDS
In this context, synthetic oligonucleotides of varying chemistry (typically 16–25 nucleotides), which are introduced into cells by various means, or simply (although inefficiently) by concentration-driven endocytosis.

ANTISENSE
Reverse complement of any DNA or RNA sequence.

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doi:10.1038/nrd837

With their promise of high specificity and low toxicity, many believe that gene-targeted therapies will lead to a revolution in cancer therapeutics[1]. Numerous gene-therapy strategies are under development, some of which use nucleic-acid-based molecules to inhibit gene expression at either the transcriptional or post-transcriptional level[2]. This strategy clearly has other potential applications, including in cardiovascular[3,4], inflammatory[5,6] and infectious diseases[7–10], as well as organ transplantation[11].

Although conceptually elegant, the prospect of using nucleic-acid molecules for treating human malignancies and other diseases remains tantalizing, but uncertain[12]. The main cause of this uncertainty is the apparent randomness with which these materials modulate the expression of their intended targets. It is a widely held view that molecule delivery, and selection of which messenger RNA sequence to physically target, are core stumbling blocks that hold up progress in the field. In this review, we recapitulate the development of nucleic-acid drugs for modulating gene expression, discuss newer strategies for solving the problems alluded to above, and detail attempts at using these molecules therapeutically. In so doing, we hope to both educate the reader who is unfamiliar with this literature, and convince those who are sceptical that this remains a viable approach to 'on demand' manipulation of gene expression.

Modulating gene expression

The notion that gene expression could be modified through the use of EXOGENOUS NUCLEIC ACIDS derives from studies by Paterson *et al.*[13], who first used single-stranded DNA to inhibit translation of a complementary RNA in a cell-free system in 1977. The following year, Zamecnik and Stephenson[14] showed that a short (13-mer) DNA oligodeoxynucleotide that was ANTISENSE to the Rous sarcoma virus could inhibit viral replication in culture. On the basis of this work, Zamecnik and Stephenson are widely credited for having first suggested the therapeutic utility of antisense nucleic acids. In the mid 1980s, the existence of naturally occurring antisense RNAs and their role in regulating gene expression was shown[15,16]. These observations were particularly important, because they lent credibility to the belief that 'antisense' was more than just a laboratory phenomenon, and encouraged belief in the hypothesis that reverse-complementary antisense nucleic acids could be used in living cells to manipulate gene expression. These seminal papers, and the thousands that have followed, have stimulated the development of technologies that use nucleic acids to manipulate gene expression. As will be discussed below, virtually all of the available methods rely on some type of nucleotide-sequence recognition for targeting

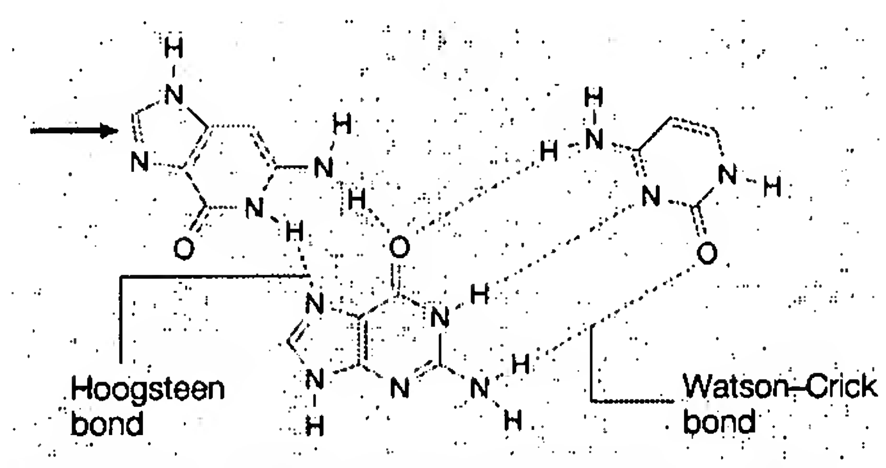

Figure 1 | **Triple-helix formation at the nucleotide level.** Shows the formation of Watson–Crick (red) and Hoogsteen bonds (black) between duplex pairs and the third strand (the arrow points to a single base of the third strand). Blue, guanine residue (purine); pink, cytosine residue (pyrimidine).

specificity, but differ as to where and how they perturb the flow of genetic information.

Strategies for modulating gene expression can be thought of as being either 'anti-gene' or anti-mRNA (see below; reviewed in REF. 2). Anti-gene strategies focus primarily on gene targeting by homologous recombination[17,18], or by TRIPLE-HELIX-FORMING OLIGODEOXYNUCLEOTIDES (TFOs)[19]. As homologous recombination involves vector technology and — at least at the present time — is much too inefficient for clinical use, it will not be considered further in this discussion. TFOs bind in the MAJOR GROOVE of duplex DNA in a sequence-specific manner[20]. Gene targeting with these molecules is constrained by the fact that TFOs require runs of purines on one strand and pyrimidines on the other (~10–30 nucleotides (nts) in length) for stable hybridization. The TFO can be composed of either polypurine or polypyrimidine tracts, but hybridization always occurs on the purine strand of the duplex through the formation of HOOGSTEEN BONDS (FIG. 1).

Successful use of this strategy for blocking transcription and inducing specific mutations, both *in vitro* and *in vivo*, has been reported (reviewed in REF. 20). Although the frequency of such events is typically <1%, Glazer and co-workers[21] have reported a system in which desired mutations can be induced in ~50% of cells, indicating that genuine clinical utility might be possible. This general approach has also been used for inducing mutations that can actually repair a gene that has been made defective by inherited or acquired point mutation. Work to support this concept using chimeric DNA–RNA oligonucleotides has also been reported, but again, the frequency of such repairs, in most cases, has been far too low to be of clinical use at this time[22].

Short, double-stranded (ds)DNA decoy molecules have also been used to disrupt gene expression at the level of transcription[23]. These oligodeoxynucleotides are designed to compete for transcription-factor complexes, with the ultimate goal of attracting them away from the promoter that they would ordinarily activate. For many technical reasons, including limited gene accessibility in the NUCLEOSOME structure, the clinical application of these methods has not progressed at a rapid rate. An alternative approach, using polyamides, or LEXITROPSINS, has been described by Dervan and colleagues[24–26]. These small molecules have the ability to diffuse into the nucleus, where they can contact dsDNA in the minor groove, thereby impeding transcription by preventing unwinding of the duplex, or by preventing the binding of transcription-factor complexes to the gene promoter. DNA accessibility, and maintaining the appropriate 'register' of the polyamides for the desired sequence recognition, are problems with this method that remain to be solved[27].

A larger body of work has focused on destabilizing mRNA. This approach, although less favourable than anti-gene strategies from a stoichiometric point of view, is nonetheless attractive, because mRNA, unlike the DNA of a given gene, is — theoretically— accessible to attack while being transcribed, transported from the nucleus or translated. Two nucleic-acid-based strategies have emerged for blocking translation. One strategy uses oligoribonucleotides. Similar to the strategy of the DNA decoys, the RNA decoys are designed to provide alternate, competing binding sites for proteins that act as translational activators or mRNA-stabilizing elements[28,29]. By attracting away the desired protein, the decoy can prevent translation, or induce instability and, ultimately, destruction of the mRNA. Recent studies on human α-globin mRNA are of interest in this regard. Stability determinants for this mRNA species have been defined in sufficient detail so that it can be used as a model system for testing the hypothesis that altering mRNA stability with decoys will be a useful form of therapy[29–31].

The other strategy for destabilizing mRNA is the more widely applied antisense strategy, which uses RIBOZYMES, DNAzymes, antisense RNA or antisense DNA (ODN). The antisense approach to modulating gene expression has been the subject of numerous authoritative reviews, and will not be discussed in great detail here[2,32,33]. Simply stated, delivering a reverse-complementary — that is, 'antisense' — nucleic acid into a cell in which the gene of interest is expressed should lead to hybridization between the antisense sequence and the mRNA of the targeted gene. Stable mRNA–antisense duplexes can interfere with the splicing of heteronuclear RNA into mature mRNA[34,35], block translation of completed message[36,37] and — depending on the chemical composition of the antisense molecule —lead to the destruction of the mRNA by binding of endogenous nucleases, such as RNaseH[38,39], or by intrinsic enzymatic activity engineered into the sequence, as is the case with ribozymes[40,41] and DNAzymes[42,43] (FIG. 2).

Nucleic acids with catalytic activity

Ribozymes and DNAzymes bind to substrate RNA through Watson–Crick base pairing, which offers sequence-specific cleavage of transcripts. At least six classes of ribozyme have been described. Two ribozymes, the 'hammerhead' ribozyme and the 'hairpin' ribozyme, have been extensively studied owing to their small size and rapid kinetics[44,45]. The catalytic motif is surrounded by flanking sequence that is responsible for 'guiding' the ribozyme to its mRNA target and giving stability to the structure. With the hammerhead ribozyme, cleavage is dependent on divalent cations, such as magnesium, and can occur after any NUH

TRIPLE-HELIX-FORMING OLIGODEOXYNUCLEOTIDE (TFO). A synthetic, single-stranded oligodeoxynucleotide, which, through Hoogsten-bond formation, hybridizes to purine/pyrymidine-rich sequences in double-stranded DNA. Formation of stable triple helices can prevent the unwinding that is necessary for transcription of the targeted region or block the binding of transcription-factor complexes.

MAJOR GROOVE AND MINOR GROOVE
Channels formed by the twisting of two complementary DNA strands around each other to form a double helix. The major groove is ~22 Å wide and the minor groove is ~12 Å wide.

HOOGSTEEN BOND
Triple-helix-forming oligonucleotides hybridize with purine bases that comprise polypurine/polypyrimidine tracks in the DNA. The hydrogen bonds that are formed under these conditions are referred to as Hoogsteen bonds after the individual who first described them. They can form in parallel or antiparallel (reverse-Hoogsteen) orientations.

NUCLEOSOME
A packing unit for DNA within the cell nucleus, which gives the chromatin a 'beads-on-a-string' structure. The 'beads' consist of complexes of nuclear proteins (histones) and DNA, and the 'string' consists of DNA only. A histone octamer forms a core around which the double-stranded DNA helix is wound twice.

LEXITROPSIN
A molecule that extragenetically reads the base sequence of double-stranded DNA.

RIBOZYME
RNA molecule that contains one of a variety of catalytic motifs that cleave RNA to which it hybridizes.

DNAzyme
A DNA molecule that contains a catalytic motif that cleaves RNA to which it hybridizes.

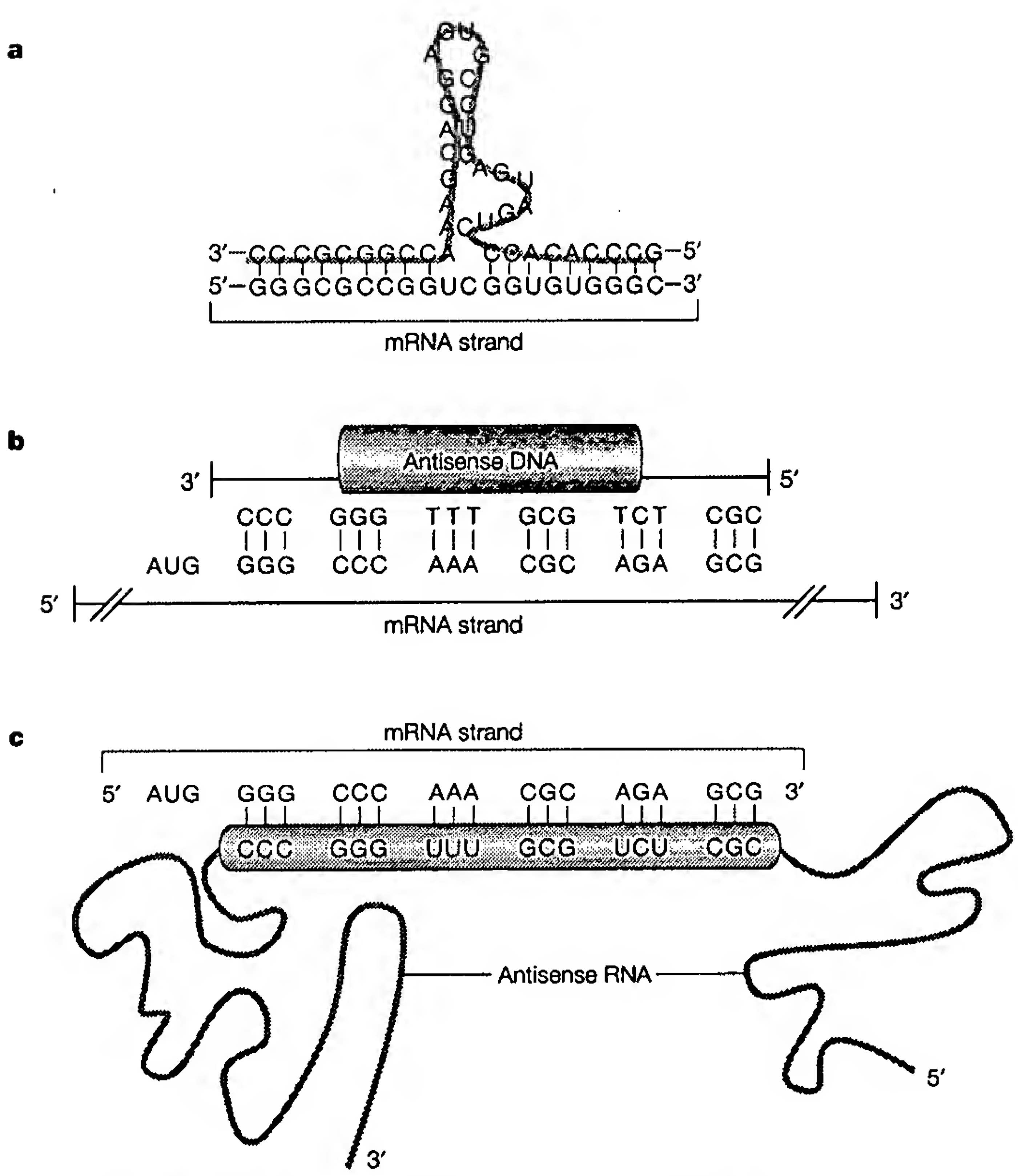

Figure 2 | **Strategies for inhibiting translation.** Diagrammatic representations of **a** | a hammerhead ribozyme (DNAzymes have similar RNA-cleaving capabilities, but the catalytic motif is composed of DNA nucleotides, hence the name); **b** | an antisense oligodeoxynucleotide; and **c** | antisense RNA. Note that targeting specificity is conveyed in each case by Watson–Crick base pairing between complementary sequences. From REF. 2 © (1998) American Society of Hematology, used by permission. mRNA, messenger RNA.

triplet within the target RNA sequence, for which 'N' represents any nucleotide, 'U' represents uracil and 'H' represents adenine, cytosine or uracil[46,47]. If ribozymes are to work effectively as 'enzymes,' they must not only bind substrate RNA but also dissociate from the cleavage product to act on further substrates. Dissociation from the cleavage product might, in fact, be an important rate-limiting step that controls their usefulness[48,49]. Consideration of reaction kinetics indicates that ribozymes might have a theoretical advantage over RNase-H-dependent antisense oligonucleotides, but to the best of our knowledge, this has not been shown consistently *in vivo*. Ribozymes can be expressed from a vector that offers the advantage of continued production of these molecules intracellularly[50,51], a property that — at least until recently — was not possible with antisense DNA[52]. However, it is well known that stable transduction of primary cells *in vivo* has substantial technical problems, which will not be discussed further. Progress has been made recently in synthesizing stable forms of these molecules, so that they might be delivered directly to cells both *in vitro* and *in vivo*[53].

DNAzymes have evolved from the seminal work of Breaker and Joyce[54], who first showed that DNA, as well as RNA molecules, could act enzymatically and cleave a nucleic-acid substrate. Similar to ribozymes, DNAzymes have a catalytic domain that is flanked by two substrate-recognition domains. After binding to their RNA substrate, DNAzymes can cleave sequences that contain purine–pyrimidine junctions. DNAzymes have some theoretical advantages over ribozymes. DNA is more stable than RNA, it is easier to synthesize, and the turnover rates for some of the DNAzymes are reported to be higher than some ribozymes[42]. Nevertheless, constant improvements in both DNAzyme[55] and ribozyme chemistry make this a 'moving target' in terms of which chemistry is better[56]. Although experience with DNAzymes as potential therapeutic agents is limited[43], these molecules might prove worthy in the clinical setting.

RNA interference

A newly developing approach for targeting mRNA is called post-transcriptional gene silencing, or RNA interference (RNAi)[57–58] (FIG. 3). RNAi is the process by which dsRNA targets mRNA for destruction in a sequence-dependent manner. The mechanism of RNAi initially involves processing of long (~500–1,000 nucleotides) dsRNA into 21–25 base-pair (bp) 'trigger' fragments[59] by a member of the RNase-III family of nucleases called DICER[60–62]. When incorporated into a larger, multicomponent nuclease complex named RISC (RNA-induced silencing complex), the processed trigger strands form a 'guide sequence' that targets the RISC to the desired mRNA sequence and promotes its destruction[61]. RNAi has been used successfully for gene silencing in various experimental systems, including petunias, tobacco plants, neurospora, *Caenorhabditis elegans*, insects, planaria, hydra and zebrafish. The use of long dsRNA to silence expression in mammalian cells has been tried, largely without success[63]. More recent reports using short interfering RNA (siRNA; see below) seem to be more promising[64]. It has been suggested that mature, as opposed to embryonic, mammalian cells recognize these long dsRNA sequences as invading pathogens. This triggers a complex host-defence reaction that effectively shuts down all protein synthesis in the cell through an interferon-inducible serine/threonine-kinase enzyme called protein kinase R (PKR). PKR phosphorylates the α-subunit of eukaryotic initiation factor-2 (EIF-2α), which globally inhibits mRNA translation. The long dsRNA also activates 2′,5′-oligoadenylate synthetase, which in turn activates RNase L. RNase L indiscriminately cleaves mRNA. Cell death is the understandable result of these processes. Recently, a number of reports have suggested that siRNA strands — RNA double strands of ~21–22 nucleotides in length — do not trigger this host-defence response, and therefore might be able to silence expression in mammalian somatic cells if appropriately modified to contain 3′-hydroxy and 5′-phosphate groups[66–68]. The universality of this approach, and the types of gene that can be modified using this strategy in mammalian cells, remain unknown at this time.

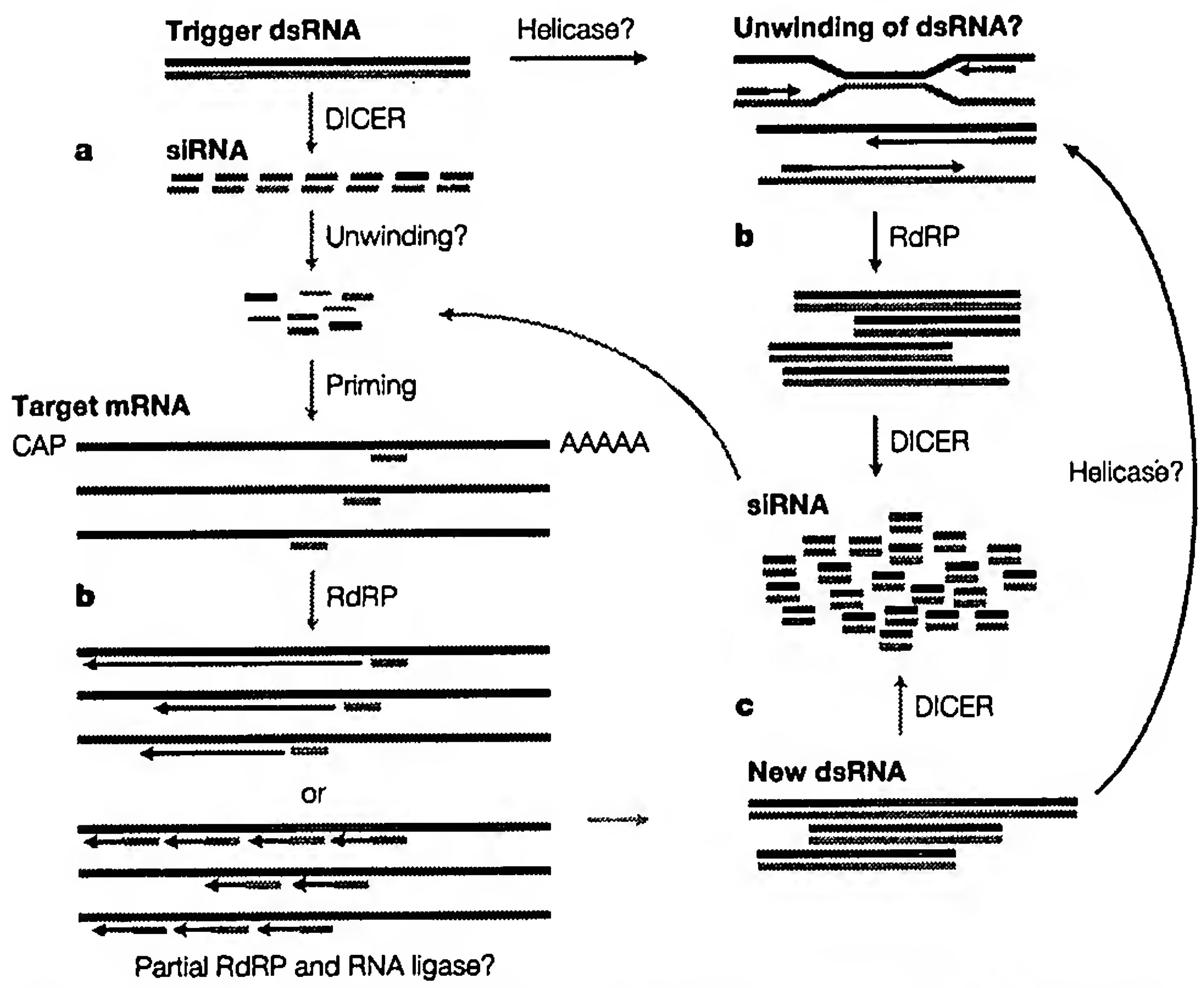

Figure 3 | **Hypothetical RNAi mechanism. a** | In the 'initiation' stage of RNA interference (RNAi), a small amount of trigger double-stranded (ds)RNA is processed into short interfering (si)RNA by an enzyme called DICER (light blue arrow), which is used as an RdRP primer. **b** | The RdRP reaction converts target messenger RNAs into new dsRNAs (next generation of trigger dsRNAs), which are then processed into new siRNAs, establishing a self-sustaining cycle of RNAi 'maintenance' (green arrows). **c** | Replication of 'trigger' or newly synthesized dsRNA by RdRP would amplify the potency of RNAi by further increasing the amount of siRNA, as both sense and antisense strands of trigger dsRNA and siRNA could then be used. However, the *in vivo* significance of this pathway (dark grey arrows) has not yet been established. It also remains unclear if the 'amplification' steps take place in mammalian cells. RdRP, RNA-dependent RNA polymerase; helicase, unwinding enzyme. Redrawn from REF. 57 © (2001), with permission from Elsevier Science.

Altering RNA splicing

Finally, the strategy of manipulating gene expression by altering RNA processing, as opposed to by mRNA destruction, is also worth mentioning, as significant progress seems to have been made in this area. Kole and colleagues developed this approach using a model system based on human thalassaemia[69,70]. Thalassaemias are highly prevalent human blood disorders that are characterized by faulty haemoglobin production and concomitant red-cell destruction that results in anaemia. The genetic mutations that are responsible for these diseases are well characterized, and often involve aberrant splicing. Kole's group showed that treatment of mammalian cells that were stably expressing a human β-globin gene with antisense oligonucleotides that were targeted at the aberrant splice sites blocked the abnormal splicing, thereby allowing the normal splice site to be used. Correction of splicing was oligo-dose dependent and, importantly, led to accumulation of normal human β-globin mRNA and polypeptide in cells[69]. More recently, correction has been accomplished in blood cells derived from thalassaemic patients[71]. This result would clearly have important clinical consequences if such treatment could be made effective at the level of the haematopoietic stem cell. These same workers suggest that this approach might also be useful in the treatment of cancer[72].

Increasing oligonucleotide stability

Initial work with antisense DNA was carried out with unmodified, natural molecules. It soon became clear, however, that native DNA was subject to relatively rapid degradation, primarily through the action of 3′ exonucleases, but also as a result of endonuclease attack. Molecules destined for the clinic, and those used for experimental purposes, are now routinely modified to enhance their stability, as well as the strength of their hybridization with RNA (see REFS 73,74 for further details). Oligonucleotide drugs need to meet certain physical requirements to make them useful. First, they must be able to cross cell membranes and then hybridize with their intended target. The ability of an ODN to form a stable hybrid is a function of its binding affinity and sequence specificity. Binding affinity is a function of the number of hydrogen bonds that are formed between the ODN and the sequence to which it is targeted. This is measured objectively by determining the temperature at which 50% of the double-stranded material is dissociated into single strands, which is known as the melting temperature, or T_m. mRNA-associated proteins and tertiary structure also govern the ability of an ODN to hybridize with its target by physically blocking access to the region that is being targeted by the ODN. Finally, it is also clear that ODNs should exert little in the way of non-sequence-related toxicity[75], and should remain stable in the extracellular and intracellular milieu in which they are situated. Meeting all these requirements in any one molecule has turned out to be a demanding task. Satisfying one criterion is often accomplished at the expense of another. It is also worth noting that the more complex the molecule, the more expensive is its synthesis. In an age of increasing cost consciousness, this too becomes an important design consideration.

First-generation antisense molecules were designed to make the internucleotide linkages — the backbone on which the nucleosides are hung — more resistant to nuclease attack. This was accomplished primarily by replacing one of the non-bridging oxygen atoms in the phosphate group with either a sulphur or a methyl group. The former modification, which is called a phosphorothioate oligodeoxynucleotide, proved highly successful, because these molecules are relatively nuclease resistant, they are charged and therefore water soluble, and they activate RNase H. All of these properties are desirable, and virtually all of the clinical trials done so far have been carried out with this chemistry, although trials using so-called 'second-generation molecules' (mixed backbone/chimeric oligonucleotides) will shortly begin. Second-generation molecules were developed to overcome the disadvantageous properties of the phosphorothioates. A primary strategy that was used was to remove the phosphorothioate linkages to the greatest extent possible. This was often done by flanking a phosphorothioate core with nuclease-resistant nucleosides — often with 2′-*O* sugar modifications — that rendered the molecules more RNA like, and therefore gave tighter binding to the target.

MORPHOLINO OLIGODEOXYNUCLEOTIDE (PMO). The base is attached to a morpholino instead of a ribofuranosyl ring, and the backbone is composed of a phosphorodiamidate linkage.

Many chemical modifications to the phosphodiester linkage have been made. Two of the more interesting modifications that are now under development are peptide nucleic acids (PNAs)[76] and MORPHOLINO OLIGODEOXYNUCLEOTIDES (PMOs)[36]. These compounds are essentially nuclease resistant. PNAs represent a more radical approach to the nuclease-resistance problem, as the phosphodiester linkage is completely replaced with a polyamide (peptide) backbone. They both form extremely tight bonds with their RNA targets and probably exert their effects by blocking translation, as neither molecule effectively activates RNase H. Whether it is necessary to preserve the ability of these molecules to activate RNase H is controversial[37], but many workers in the field still believe that molecules with this capability are likely to be more effective, at least in the clinical setting. As these molecules do not move freely across cell membranes, they must be injected or transfected into cells. Finally, PNAs are also sensitive to local ionic concentration and do not hybridize as well under physiological conditions.

Nucleic-acid drugs in the clinic

Diseases that are characterized by overexpression or inappropriate expression of specific genes, or genes that are expressed by invading microorganisms, are candidates for gene-silencing therapies. For this reason, the earliest clinical trials with these agents have been against human immunodeficiency virus (HIV)77–79 and patients with cancer80. Malignant diseases, in particular, are attractive candidates for this therapeutic approach, if for no other reason than that conventional cancer therapies are highly toxic. As antisense strategies are directed against genes that are aberrantly expressed in diseased cells, it might reasonably be expected that this approach will engender fewer and less serious side effects, as normal cells should not be affected. There were concerns that this might not be the case when preclinical studies on primates with phosphorothioate compounds resulted in the death of some animals. However, investigation of these occurrences showed that they took place after rapid bolus intravenous infusions at concentrations exceeding 5–10 μg ml^{-1}, and that they were probably due to complement activation and vascular collapse[81]. This experience was therefore a useful reminder that, in addition to side effects resulting from the suppression of the targeted gene, side effects related to the chemical backbone of the oligonucleotide should also be anticipated. In the case of phosphorothioates, this problem was easily addressed by infusing material continuously, or slowly, and at lower doses. In actual use in the clinic, phosphorothioates have proved to be remarkably well tolerated (BOX 1). Abnormalities related to the backbone include transient fever, fatigue, nausea and vomiting, mild to moderate thrombocytopaenia and transient prolongation of partial thromboplastin time (PTT; 1.25–1.75 ×), which is fortunately unassociated with any signs of overt clinical bleeding[82–85]. At present, several clinical studies have been carried out using a number of different oligonucleotides. Below, we review some of the more recent clinical studies that have been carried out on patients with malignant, inflammatory, cardiac and infectious diseases (summarized in TABLE 1).

Targeting apoptosis inhibitors in oncology

BCL2: cancer treatment. Targeting B-cell lymphoma protein 2 (BCL2) is a promising example of triggering apoptosis in tumour cells. BCL2 is an important regulator of programmed cell death, and its overexpression has been implicated in the pathogenesis of some lymphomas[86]. Resistance to chemotherapy, at least *in vitro*, might also be related to *BCL2* overexpression[87,88]. Laboratory studies have shown convincingly that exposing cells to an oligonucleotide targeted to *BCL2* will specifically decrease the amount of targeted mRNA and protein (six–eightfold reduction). For all of these reasons, there is a great deal of interest in targeting *BCL2* for therapeutic purposes[89]. Several clinical trials with a *BCL2*-targeted antisense molecule have been reported, both alone[83,90] and with supplementary chemotherapy[84,91,92]. Studies with the oligonucleotide alone have not shown consistent, strong antitumour responses. The addition of chemotherapy might be helpful in this regard. An issue with several of these studies is lack of correlation of tumour responses with significant effects on BCL2 protein expression. The mechanism of action of the compound is not entirely clear.

Transcription-factor targeting in oncology

c-MYB: bone-marrow purging. The normal homologue of the avian myeloblastosis virus oncogene (v-*myb*) is a proto-oncogene called c-*MYB*. c-*MYB* encodes a protein (MYB), which is a regulator of cell-cycle transition and cellular maturation, primarily in haematopoietic cells, but in other cell types as well. A recently published study was designed to test the hypothesis that an effectively delivered, appropriately targeted ODN might provide a proof of concept about the ability to target a specific mRNA and thereby kill tumour cells selectively[93]. To test this hypothesis, an ODN targeted to the c-*MYB* proto-oncogene was used to purge marrow autografts that were administered to patients with allograft-ineligible chronic myelogenous leukaemia (CML). $CD34^+$ marrow cells were purged

Box 1 | **First approved nucleic-acid drug**

Vitravene (sodium fomivirsen), an antiviral drug that was developed by ISIS Pharmaceuticals and is marketed by CIBAVision, was approved by European and US regulatory authorities in July 1999 and August 1998, respectively. Vitravene is used to treat an inflammatory viral infection of the eye (retinitis) that is caused by the cytomegalovirus (CMV). CMV often infects immunocompromised patients, and patients with uncontrolled AIDS are particularly at risk. One or both eyes can be affected, and it is not unusual for patients to suffer severe visual impairment or blindness as a result of untreated infections. Treatment of CMV retinitis is problematic, in particular for patients who cannot take, do not respond or become resistant to standard antiviral treatments for CMV infections, such as ganciclovir, foscarnet and cidofovir[145]. Vitravene is an antisense phosphorothioate 21-mer oligonucleotide has a sequence that is complementary to messenger RNA that is transcribed from the main immediate–early transcriptional unit of CMV[145,146].

with ODN for either 24 ($n = 19$) or 72 ($n = 5$) hours (FIG. 4). Post-purging, c-*MYB* mRNA levels declined substantially in ~50% of patients. Analysis of *BCR–ABL* (breakpoint cluster region–Abelson murine leukaemia viral oncogene homologue) expression in a surrogate stem-cell assay indicated that purging had been accomplished at a primitive cell level in >50% of patients. Cytogenetics were evaluated at day 100 in surviving patients who did not require administration of unpurged 'rescue' marrow for engraftment ($n = 14$). (All purging protocols require storage of untreated marrow as a 'back-up', in case the purged material does not engraft.) Whereas all patients were ~100% Ph$^+$ (Philadelphia chromosome positive) pre-transplant, two patients had complete cytogenetic remissions, three patients had <33% Ph$^+$ metaphases and eight remained 100% Ph$^+$. The marrow of one patient yielded no metaphases, but fluorescence *in situ* hybridization (FISH) evaluation ~18 months post-transplant revealed that ~45% of cells were *BCR–ABL*$^+$, indicating that six

Table 1 | **Summary of recently published clinical trials with nucleic-acid drugs**

Target	Type of study	No. of patients	Diagnosis	Dose range	Treatment duration	Administration	Remissions	Refs
ICAM-1	Multicentre; placebo controlled; double blind	75	Crohn's disease	0.5 mg	2 days–4 weeks	SC	Not significant	106
	Placebo controlled; double blind	20	Crohn's disease	0.5–2 mg kg^{-1}	26 days	2 hours IV infusion	47% steroid-free remissions	6
PKC-α	Phase I	36	Advanced cancer	0.15–6 mg kg^{-1}d^{-1}	3 days per week for 3 weeks every 4 weeks	2 hours IV infusion	2 CR	82
	Phase I	21	Advanced cancer	0.5–3 mg kg^{-1}d^{-1}	21 days every 4 weeks	Continuous IV infusion	3 responses	85
BCL2	Phase I	21	Relapsed NHL	4.6–195.8 mg m^{-2} d^{-1}	14 days	Continuous SC infusion	1 CR, 2 minor responses	83
BCL2 combined with dacarbazine	Phase I/II	14	Advanced malignant melanoma	0.6-6.5 mg kg d^{-1}	14 days every 4 weeks	Continuous IV infusion	1 CR, 2 PR 3 minor responses	91
BCL2 combined with mitoxantrone	Phase I/II	26	Metastatic prostate cancer	0.6–5 mg kg^{-1} d^{-1}	14 days every 28 days	Continuous IV infusion	2 decreases in PSA	84
Fomivirsen CMV	Multicentre; randomized; prospective	29	CMV retinitis in AIDS patients	165 μg	Once per week	Intravitreously	Time to progression 71 versus 13 days	147
h-RAS	Phase I	23	Advanced cancer	0.5–10 mg kg^{-1} d^{-1}	14 days every 3 weeks	Continuous IV infusion	4 stable	96
c-RAF kinase	Phase I	34	Advanced cancer	1–5 mg kg^{-1} d^{-1}	21 days every 4 weeks	Continuous IV infusion	2 stable diseases	119
	Multicentre Phase II	22	SCLC and NSCLC	2 mg kg^{-1} d^{-1}	21 days every 4 weeks	Continuous IV infusion	No responses	148
	Phase I	22	Advanced cancer	6–30 mg kg^{-1} d^{-1}	Weekly	24 hours IV infusion	No responses	99
c-MYC	Multicentre; placebo controlled	78	After PTCA	1–24 mg d^{-1}	Single dose	intracoronary	No responses	108
	Placebo controlled	85	After coronary-stent implantation	10 mg d^{-1}	Single dose	Intracoronary	No responses	109
IGF1R	Pilot study	12	Malignant astrocytoma	2 mg 10^{-7} cells	6 hours	*Ex vivo*	2 CR 6 PR	118

AS, antisense; BCL2, B-cell lymphoma protein 2; CMV, cytomegalovirus; CR, complete remission; ICAM-1, intercellular adhesion molecule-1; IGF1R, insulin-like-growth-factor-1 receptor; IV, intravenous; c-MYC, myelocytomatosis viral oncogene homologue; NHL, non-Hodgkin's lymphoma; NSCLC, non-small-cell lung cancer; PKC-α; protein kinase C-α; PR, partial remission; PSA, prostate-specific antigen; PTCA, percutaneous transluminal coronary angioplasty; SC, subcutaneous; SCLC, small-cell lung cancer.

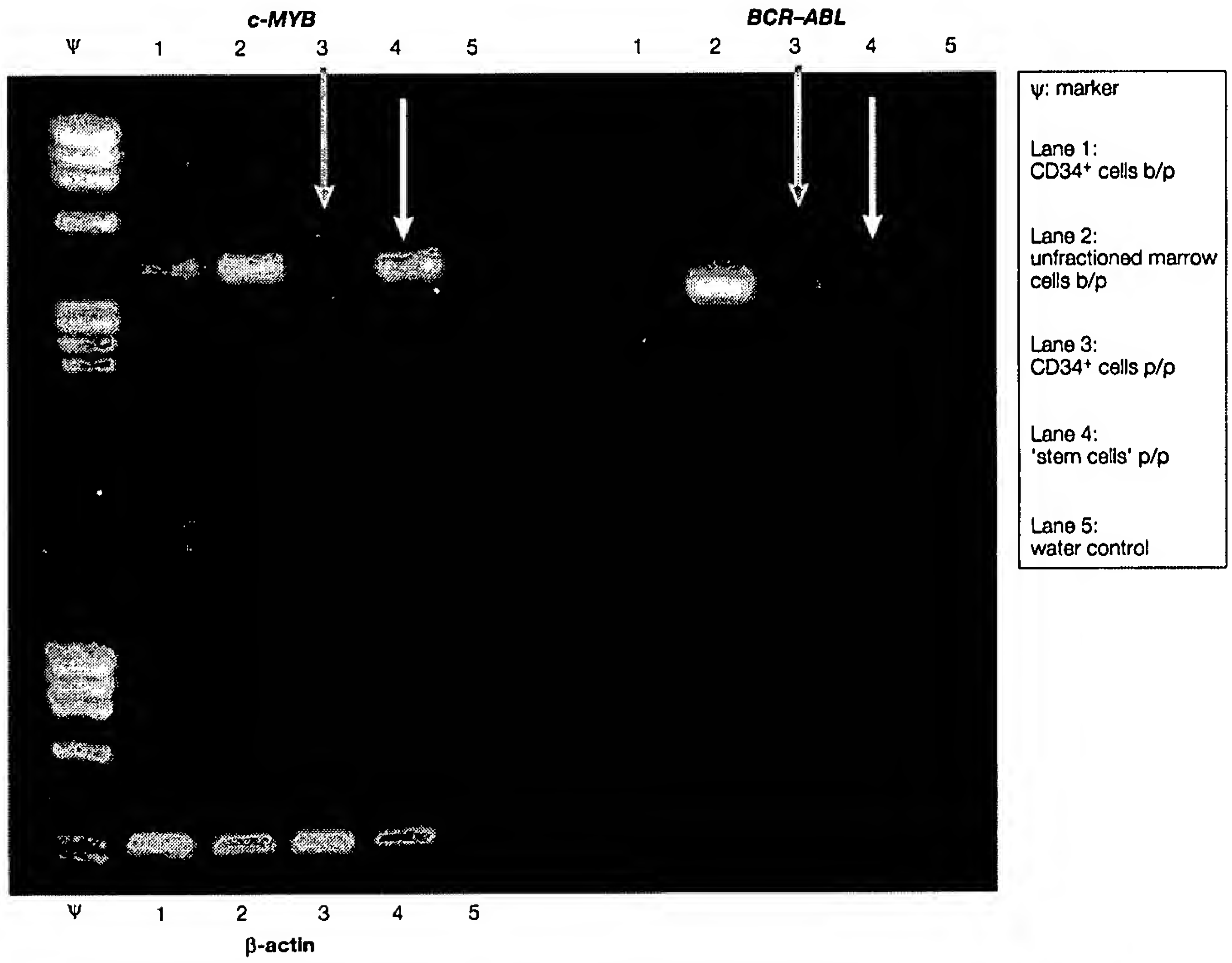

Figure 4 | **Effect of c-*MYB*-targeted ODNs on c-*MYB* mRNA expression in marrow cells.** Ethidium-bromide-stained agarose gel containing c-*MYB*, *BCR–ABL* and β-actin messenger RNA reverse transcriptase (RT)-PCR products derived from: CD34⁺ bone-marrow cells of a representative patient before anti-c-*MYB* oligodeoxynucleotide purging (Lane 1); unfractionated bone-marrow cells before purging (Lane 2); CD34⁺ cells post-purging (Lane 3); and the patient's primitive 'stem cells' post purging (Lane 4). A control RT-PCR reaction that contains only water is shown in Lane 5. Lanes containing molecular-weight markers are indicated by the symbol Ψ. Lane 3 (orange arrows) reveals that c-*MYB* mRNA is undetectable post purging, whereas some residual *BCR–ABL* expression (molecular marker of the malignant cells) persists. Efficiency of the process on primitive haematopoietic cells is shown in lane 4 (white arrows). Here, stem cells, cultured for ten days post-purge, show normal MYB expression, whereas *BCR–ABL* expression is undetectable. These data indicate that, in this patient's marrow sample, normal cells survived the purge but malignant, *BCR–ABL*-expressing cells did not. Control cells that were treated in an identical manner but not exposed to the anti-c-*MYB* oligodeoxynucleotide continue to express *BCR–ABL* (not shown), which indicates that the results are due to oligodeoxynucleotide exposure and are not a cell-culture artefact. b/p, before purging; p/p, post purging.

out of fourteen patients had originally obtained a 'major' cytogenetic response. Conclusions about clinical efficacy of ODN marrow purging could not be drawn from this small pilot study. Nevertheless, these results led the authors to speculate that enhanced delivery of ODN, targeted to crucial proteins with short half-lives, might lead to the development of more effective nucleic-acid drugs and enhanced clinical utility of these compounds in the future.

Oncogenic signal-transduction pathways

Protein kinase C-α. Protein kinase C (PKC) comprises a family of biochemically and functionally distinct phospholipid-dependent, cytoplasmic serine/threonine kinases. These proteins have a crucial role in transducing the signals that regulate cell proliferation and differentiation. PKC is overexpressed in several tumours, and antisense inhibitors of these enzymes have shown some antitumour activity *in vitro*[5,82,94] and in animal models[95]. Results of two studies that used the identical 20-mer phosphorothioate ODN against *PKCα* have been published[82,85]. The ODN was well tolerated, but antitumour effects were modest at best. Correlations with levels of *PKCα* expression were not provided.

RAS pathway

h-RAS oligonucleotide. h-RAS is a powerful regulator of several interconnected receptor-signalling pathways. The gene is constitutively active, and promotes proliferation and malignant transformation in many human tumours. Cunningham *et al.* reported results from a study that was carried out on 23 patients with various malignancies[96]. As in other studies with phosphorothioate oligonucleotides, only mild toxicities were observed. No complete or partial responses were achieved. Four patients had stabilized disease for 6–10 cycles of treatment.

c-RAF kinase. RAF proteins are crucial effectors in the RAS signal-transduction pathway. Constitutive activation of the RAS pathway is thought to contribute to malignant transformation in many cell types, which makes elements of this signalling pathway attractive targets for inhibition. Effectiveness of an antisense oligonucleotide against c-*RAF* has been shown both *in vitro*[97] and in an *in vivo* tumour-xenograft model[98]. On the basis of this work, three clinical trials were initiated[99,119,148]. A total of 78 patients were treated. No major tumour responses were documented, but some patients had stabilization of their disease.

Ribozymes

Ribozymes have been the subject of several authoritative reviews[41,100]. Although there is a comprehensive literature that describes the use of these molecules to target a wide variety of mRNA species in various cell-free, cell-intact and animal-model systems (see REFS 41,111), there is little recently published material on the use of these materials in clinical trials. The earliest clinical use of ribozymes was in patients with HIV[77,78,101,102]. As is true of antisense oligodeoxynucleotides, the approach was found to be safe when ribozymes were expressed in cells that were then delivered back to patients, but clinical efficacy was found wanting. At present, several Phase I/II clinical trials with exogenously delivered synthetic ribozymes are in early-phase clinical evaluation for patients with breast cancer, colon cancer and hepatitis. Results of these clinical investigations are anxiously awaited.

Studies in non-malignant diseases

Inflammatory diseases. Antisense oligonucleotides have been explored as anti-inflammatory agents. An example is the targeting of intracellular adhesion molecule-1 (ICAM-1) in Crohn's disease. In response to inflammatory stimuli, many cells upregulate the expression of *ICAM1*, which has an important role in the transport and activation of leukocytes. It has been shown *in vitro* and *in vivo* that administration of antisense oligonucleotides against *ICAM1* causes a decrease in receptor expression, which in turn ameliorates inflammatory reactions[103–105]. Two clinical trials with this compound in patients with Crohn's disease have been reported[6,106]. In the double-blind study reported by Yacyshyn *et al.*[6], 20 patients were randomized to receive a saline placebo or anti-*ICAM1* antisense oligonucleotide. The treatment was well tolerated, and after 6 months, disease remission was reported in 47% of patients in the antisense group compared with 20% of patients in the placebo group. Furthermore, corticosteroid usage was significantly lower ($p = 0.0001$) in the antisense-treated patients. These results engendered a great deal of excitement, but the enthusiasm was subsequently dampened by the follow-on study that was carried out with this compound in a larger group of patients with this disease ($n = 75$)[106]. In this placebo-controlled study, no statistically significant differences in steroid use between the treatment or placebo groups was observed, although 'positive trends' were seen in the patients who were treated with the antisense oligonucleotide. As with other studies, toxicity was mild and consisted primarily of pain at the injection site, fever and headache.

The anti-*ICAM1* oligonucleotide has also been evaluated in patients with psoriasis. The drug was initially administered by intravenous infusion to these individuals, but examination of their skin indicated that delivery to its various layers was poor. For this reason, a topical formulation was developed. Although preclinical data about uptake of this formulation into the skin and downregulation of expression of the target were encouraging[107], the ensuing clinical trial showed only modest, short-term effects in these patients (see the ISIS Pharmaceuticals web site online). The ultimate usefulness of this compound remains to be determined.

Cardiovascular disease. RESTENOSIS of coronary vessels after *trans*-catheter re-vascularization procedures remains a serious clinical problem. Manipulation of coronary vessels invariably leads to endothelial-cell injury, which is often accompanied by thrombosis, smooth-muscle-cell activation and subsequent vascular remodelling. The myelocytomatosis viral oncogene homologue (c-MYC) has been identified as an important mediator in this process through its effects on regulating the growth of vascular cells in atherosclerotic lesions. Accordingly, it has been postulated that c-*MYC* might make an attractive target for preventing post-angioplasty complications, and at least two clinical trials using a 15-mer phosphorothioate-modified antisense ODN against c-*MYC* have been reported[108,109]. Both studies showed safety of intracoronary application of the drug, but no objective clinical responses.

Oligonucleotides as immunological adjuvants

Over the past several years, it has become increasingly appreciated that several types of immune cell have pattern-recognition receptors that can distinguish prokaryotic DNA from vertebrate DNA[110]. This is apparently accomplished by the ability of these receptors to recognize unmethylated CpG dinucleotides in certain base contexts (CpG motifs)[111]. Bacterial DNA, or more germane to this discussion, synthetic oligodeoxynucleotides that contain these unmethylated CpG motifs, can activate immune responses that have evolved to protect the host against infections. Responses of this type are similar to T-helper type 1 (T_H1)-cell responses, and lead to activation of natural killer (NK) cells, dendritic cells, macrophages and B cells[112]. CpG DNA-induced immune activation has been shown to protect certain hosts against infection, either alone, or in combination with vaccines. It is reasonable to suppose, then, that CpG-containing oligonucleotides might prove to be effective adjuvants for the immunotherapy of cancer, and for boosting immune responses to antigens that are less efficient in this regard, but to which one would like to immunize a host[113].

The most recent application of this principle was reported in abstract form at the December 2001 meeting of the American Society of Hematology, where preliminary results from a clinical trial in which the

RESTENOSIS
A reduction in lumenal size after an inter-arterial coronary intervention.

Table 2 | **Current and planned clinical trials with antisense oligonucleotides and ribozymes**

Product	Diseases	Company
Anti-c-MYC (AS)	Cardiovascular restenosis, Phase II	AVI Biopharma
EPI 2010 (AS against adenosine A1 receptor)	Asthma, Phase II	EpiGenesis Pharmaceuticals
Genasense (AS against BCL2)	Haematological malignancies Solid tumours, Phase III	Genta
GTI 2040 (AS against ribonucleotide reductase)	Solid tumours, Phase I and II	Lorus Therapeutics
HGTV (AS against HIV)	HIV, Phase II	Enzo Biochem
CpG molecules	Solid tumors Infectious diseases, Phase I/II	Coley Pharmaceutical Group
Angiozyme (Ribozyme against VEGFR1)	Breast and colon cancer, Phase II	Ribozyme Pharmaceuticals
Heptazyme (Ribozyme against HCV)	HCV, Phase I	
Herzyme (Ribozyme against HER2)	Breast and ovarian cancer, Phase I	
ISIS 3521 (PKC-α) ISIS 5132 (c-RAF) ISIS 2503 (h-RAS) G 3139 (BCL2) GEM 231 (PKA)	NSCLC, NHL, Phase III Solid tumours, Phase II NSCLC, Phase II NHL, Phase II/III PKA, Phase II	ISIS Pharmaceuticals

AS, antisense; BCL2, B-cell lymphoma protein 2; CpG, unmethylated CpG dinucleotides; HCV, hepatitis C virus; HER2, tyrosine-kinase growth-factor receptor, also called c-ERBB2; HIV, human immunodeficiency virus; c-MYC, myelocytomatosis viral oncogene homologue; NHL, Non-Hodgkin's lymphoma; NSCLC, non-small-cell lung cancer; PKA, protein kinase A; PKC-α; protein kinase C-α; VEGFR1, vascular-endothelial-growth-factor receptor 1.

safety and efficacy of a CpG adjuvant was investigated in 16 patients with non-Hodgkin's lymphoma were reported[114]. Analysis of the data accrued at the time of submission indicated that the oligonucleotide increased the number and activity of NK cells in treated patients, and 2 out of 16 treated patients achieved partial remission. The study is continuing, and a follow-on trial of the CpG oligonucleotide in combination with rituximab is being planned.

Problems in need of solution

Nucleic-acid-mediated gene silencing has been used with great success in the laboratory[107,115–117], and this strategy has also generated some encouraging results in the clinic[90,93,96,118,119]. Nevertheless, it is widely appreciated that the ability of nucleic-acid molecules to modify gene expression *in vivo* is quite variable, and therefore wanting in terms of reliability[120,121]. Several issues have been implicated as a root cause of this problem, including molecule delivery to targeted cells and specific compartments within cells, and identification of sequence that is accessible to hybridization in the genomic DNA or RNA[2]. Intuitively, DNA accessibility is limited by compaction of nuclear material and transcription activity of the gene target. Formal approaches for solving this problem have not been widely discussed. In mRNA, sequence accessibility is dictated by internal base pairing and the proteins that associate with the RNA in a living cell. Attempts to accurately predict the *in vivo* structure of RNA have been fraught with difficulty[122]. Accordingly, mRNA targeting is largely a random process, which accounts for the many experiments in which the addition of an antisense nucleic acid yields no effect on expression. Several approaches to this problem have been tried, including trial-and-error 'walks' down the mRNA[123], computer-assisted modelling of RNA structure[124], hybridization of RNA to random oligonucleotides arrayed on glass slides[125,126] and variations on the theme of using random oligonucleotide libraries to identify RNase H cleavable sites, in the absence or presence of crude cellular extracts[127,128]. Recent work from this laboratory indicates that self-quenching reporter molecules might be useful for solving *in vivo* RNA structure[129], but the reliability and usefulness of this approach remain to be proven.

Another problem in this field is the limited ability to deliver nucleic acids into cells and have them reach their target[120]. Without this ability, it is clear that even an appropriately targeted sequence is not likely to be efficient. As a general rule, oligonucleotides are taken up primarily through a combination of adsorptive and fluid-phase endocytosis[130,131]. After internalization, confocal and electron microscopy studies have indicated that the bulk of the oligonucleotides enter the endosome–lysosome compartment, in which most of the material becomes either trapped or degraded. Biological inactivity is the predictable consequence of these events. Nevertheless, oligonucleotides can escape from the vesicles intact, enter the cytoplasm and then diffuse into the nucleus, where they presumably acquire their mRNA, or in the case of decoys, protein target[131,132–134]. Delivery technologies continue to improve, so it is likely that present methods, and/or other evolving technologies, will be used successfully to deliver optimized nucleic acids to their cellular targets[135,136]. Indeed, it is our hypothesis that development of

effectively targeted and efficiently delivered nucleic-acid molecules will lead to important advances in the diagnosis and treatment of human malignancies[93], and other diseases for which this class of molecule has been proposed to be effective.

In addition to delivering and targeting oligonucleotides to the mRNA, we believe that other considerations might improve the efficacy of this strategy. In this regard, we suggest that the abundance and half-life of the target mRNA should also be considered when selecting a gene target. The c-*MYB* mRNA that we have chosen to target, as well as its encoded protein, has an estimated half-life of ~30–50 minutes[137,138]. By contrast, BCL2, for example, has a half-life that has been estimated at ~14 hours[139], and RAF and RAS have half-lives that are estimated to be >24 hours[140,141]. Attempts to eliminate these proteins from cells using oligonucleotides might therefore prove more difficult. Whether these considerations will apply to extremely long lived or endogenously expressed antisense vectors, remains to be seen. As the efficiency of these molecules for perturbing gene expression improves, an important consideration in target selection will be the relative selection in the target versus non-targeted tissue. The ability to target genetic polymorphisms, or cells affected by loss of heterozygosity, might be an effective solution to this problem[142]. Finally, another approach for improving the effectiveness of nucleic-acid drugs as anticancer agents that is under intense investigation is to combine them with more traditional therapeutic modalities. Although this might well prove useful, we strongly believe that it remains important to continue to explore strategies that are designed to promote more reliable and efficient gene silencing with oligonucleotides alone. As discussed above, a prime motivating force for developing these drugs is the hope for non-toxic therapies. Adding back chemotherapy, although perhaps useful in the short term, is in the end counter-productive to this specific goal, unless it can be used at significantly reduced dosages. So far, this has not been the case.

Conclusions

The concept of inhibiting gene expression with antisense nucleic acids developed from studies that were initiated almost a quarter of a century ago[13,14]. Despite the fact that the mechanism by which these molecules modulate gene expression is not always certain[12,128,143], clinical development of antisense compounds has proceeded to the point at which several nucleic-acid drugs have entered Phase I/II, and in a few cases, Phase III trials. Others are about to begin, or are in the late planning stages (TABLE 2). The original motivation for developing these molecules remains strong. The recent development of leukaemia cells that are resistant to the small-molecule inhibitor Gleevec provides another incentive. Although a cell might be able to evolve mutated proteins that evade a small-molecule protein inhibitor, this cannot happen if the mRNA that encodes that protein is no longer made. Accordingly, although only one antisense drug has received FDA approval so far[144], all of the investigators who have laboured long and hard in this field hope that the time to celebrate significant achievements in the clinic will shortly be forthcoming.

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Acknowledgements
This work is supported by a grant from the National Institutes of Health. A.M.G. is a Distinguished Clinical Scientist of the Doris Duke Charitable Foundation. The editorial assistance of E. R. Bien and M. Goodrum is gratefully acknowledged.

Online links

DATABASES
The following terms in this article are linked online to:
Cancer.gov: http://www.cancer.gov/cancer_information/
breast cancer | chronic myelogenous leukaemia | colon cancer | non-Hodgkin's lymphoma | NSCLC | ovarian cancer
LocusLink: http://www.ncbi.nlm.nih.gov/LocusLink/
ABL | adenosine A1 receptor | BCL2 | BCR | CD34 | DICER | α-globin | β-globin | haemoglobin | HER2 | ICAM-1 | IGF1R | c-*MYB* | c-MYC | 2',5'-oligodenylate synthetase | PKA | PKC | PKCα | PKR | c-RAF | ribonucleotide reductase | RNase H | RNase L | VEGFR1
Medscape DrugInfo:
http://promini.medscape.com/drugdb/search.asp
cidofovir | foscarnet | ganciclovir | Gleevec | rituximab | Vitravene
OMIM: http://www.ncbi.nlm.nih.gov/Omim/
Crohn's disease | psoriasis

FURTHER INFORMATION
Encyclopedia of Life Sciences: http://www.els.net/
antisense nucleic acids in biotechnology
American Society of Hematology: http://www.hematology.org/
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Access to this interactive links box is free online.

Tumor Cell Lines by Disease

ATCC has approximately 1,100 tumor cell lines from a variety of species. This list includes tumor cells from primary sites or from sources in which the primary/metastatic status is unknown. They are listed by disease state, which is described with varying degrees of specificity (ATCC reports the description provided by the depositor). Cell lines that are known to be from metastatic sites are given in the following list starting on page 170.

For more information on a cell line, see the main list starting on page 30 or use the catalog number to find the entry in the cell biology section of the ATCC online catalog.

Disease	Source	Species	Name	ATCC® No.
Adenocarcinoma	cervix	human	HeLa	CCL-2
Adenocarcinoma	cervix	human	HeLa 229	CCL-2.1
Adenocarcinoma	cervix	human	HeLa S3	CCL-2.2
Adenocarcinoma	cervix	human	H1HeLa	CRL-1958
Adenocarcinoma	cervix	human	Hs 588.T	CRL-7850*
Adenocarcinoma	cervix	human	GH329	CRL-13002†
Adenocarcinoma	cervix	human	GH354	CRL-13003†
Adenocarcinoma	cervix	human	HeLa NR1	CRL-13011†
Adenocarcinoma	colon	human	NCI-H548	CCL-249
Adenocarcinoma	colon	human	Hs 255.T	CRL-7213*
Adenocarcinoma	colon	marmoset	TAC-1	CRL-10632†
Adenocarcinoma	duodenum	human	HuTu 80	HTB-40
Adenocarcinoma	intestine, small	rat	IA-XsSBR	CRL-1677
Adenocarcinoma	kidney	human	A704	CRL-7911*
Adenocarcinoma	kidney	human	A-704	HTB-45
Adenocarcinoma	lung	human	NCI-H1373	CRL-5866
Adenocarcinoma	lung	human	NCI-H1395	CRL-5868
Adenocarcinoma	lung	human	Hs 618.T	CRL-7380*
Adenocarcinoma	lung	human	SK-LU-1	HTB-57
Adenocarcinoma	lung	human	HCC2935	CRL-2869
Adenocarcinoma	lung	human	HCC4006	CRL-2871
Adenocarcinoma	lung	human	HCC827	CRL-2868
Adenocarcinoma	mammary gland	human	Hs 274.T	CRL-7222*
Adenocarcinoma	mammary gland	human	Hs 280.T	CRL-7226*
Adenocarcinoma	mammary gland	human	Hs 281.T	CRL-7227*
Adenocarcinoma	mammary gland	human	Hs 343.T	CRL-7245*
Adenocarcinoma	mammary gland	human	Hs 362.T	CRL-7253*
Adenocarcinoma	mammary gland	human	Hs 739.T	CRL-7477*
Adenocarcinoma	mammary gland	human	Hs 741.T	CRL-7480*
Adenocarcinoma	mammary gland	mouse	JC	CRL-2116
Adenocarcinoma	mammary gland	rat	13762 MAT B III	CRL-1666
Adenocarcinoma	mammary gland	rat	NMU	CRL-1743
Adenocarcinoma	mammary gland	rat	RBA	CRL-1747
Adenocarcinoma	mammary gland	rat	SMT/2A LNM	CRL-6602*
Adenocarcinoma	ovary	human	Caov-3	HTB-75
Adenocarcinoma	pancreas	human	BxPC-3	CRL-1687
Adenocarcinoma	pancreas	human	HPAF-II	CRL-1997

* Part of the NBL collection; see page 12. † Patent item; see page 12.
See the ATCC online catalog for the complete description of a cell line.
These products are for laboratory research use only. Not intended for use in humans, animals or for diagnostics.

Tumor Cell Lines by Disease

Disease	Source	Species	Name	ATCC® No.
Adenocarcinoma	pancreas	human	HPAC	CRL-2119
Adenocarcinoma	pancreas	human	Panc 03.27	CRL-2549
Adenocarcinoma	pancreas	human	Panc 08.13	CRL-2551
Adenocarcinoma	pancreas	human	Panc 02.03	CRL-2553
Adenocarcinoma	pancreas	human	Panc 02.13	CRL-2554
Adenocarcinoma	pancreas	human	Panc 04.03	CRL-2555
Adenocarcinoma	pancreas	human	Panc 05.04	CRL-2557
Adenocarcinoma	pancreas	human	Capan-2	HTB-80
Adenocarcinoma	pancreas	mouse	LTPA	CRL-2389
Adenocarcinoma	prostate	mouse, transgenic	TRAMP-C1	CRL-2730
Adenocarcinoma	prostate	mouse, transgenic	TRAMP-C2	CRL-2731
Adenocarcinoma	prostate; transfected	human	CA-HPV-10	CRL-2220
Adenocarcinoma	rectum	human	SW837	CCL-235
Adenocarcinoma	salivary gland, submandibular	mouse	WR21	CRL-2189
Adenocarcinoma	unknown	human	TE 206.T	CRL-7758*
Adenocarcinoma	uterus, endometrium	human	KLE	CRL-1622
Adenocarcinoma	uterus, endometrium	human	HEC-1-A	HTB-112
Adenocarcinoma	uterus, endometrium	human	HEC-1-B	HTB-113
Adenocarcinoma, bronchogenic	lung	human	Hs 229.T	CRL-7194*
Adenocarcinoma, colorectal	cecum	human	NCI-H716	CCL-251
Adenocarcinoma, colorectal	cecum	human	NCI-H747	CCL-252
Adenocarcinoma, colorectal	cecum	human	NCI-H508	CCL-253
Adenocarcinoma, colorectal	cecum	human	NCI-H498	CCL-254
Adenocarcinoma, colorectal	colon	guinea pig	GPC-16	CCL-242
Adenocarcinoma, colorectal	colon	human	WiDr	CCL-218
Adenocarcinoma, colorectal	colon	human	COLO 320DM	CCL-220
Adenocarcinoma, colorectal	colon	human	COLO 320HSR	CCL-220.1
Adenocarcinoma, colorectal	colon	human	DLD-1	CCL-221
Adenocarcinoma, colorectal	colon	human	HCT-15	CCL-225
Adenocarcinoma, colorectal	colon	human	SW480	CCL-228
Adenocarcinoma, colorectal	colon	human	SW403	CCL-230
Adenocarcinoma, colorectal	colon	human	SW48	CCL-231
Adenocarcinoma, colorectal	colon	human	SW1116	CCL-233
Adenocarcinoma, colorectal	colon	human	SW948	CCL-237
Adenocarcinoma, colorectal	colon	human	SW1417	CCL-238
Adenocarcinoma, colorectal	colon	human	LS123	CCL-255
Adenocarcinoma, colorectal	colon	human	LS 180	CL-187
Adenocarcinoma, colorectal	colon	human	LS 174T	CL-188
Adenocarcinoma, colorectal	colon	human	C2BBe1	CRL-2102
Adenocarcinoma, colorectal	colon	human	Hs 257.T	CRL-7214*
Adenocarcinoma, colorectal	colon	human	Hs 587.Int	CRL-7352*
Adenocarcinoma, colorectal	colon	human	Caco-2	HTB-37
Adenocarcinoma, colorectal	colon	human	HT-29	HTB-38
Adenocarcinoma, colorectal	rectum	human	SW1463	CCL-234
Adenocarcinoma, colorectal	rectum	human	Hs 200.T	CRL-7159*
Adenocarcinoma, colorectal	rectum	human	Hs 219.T	CRL-7184*
Adenocarcinoma, ductal; cystic fibrosis	pancreas	human	CFPAC-1	CRL-1918
Adenocarcinoma, ductal	pancreas	human	PL45	CRL-2558
Adenocarcinoma, gastric	stomach	human	AGS	CRL-1739
Adenocarcinoma, ileocecal colorectal	colon	human	HCT-8 (HRT-18)	CCL-244
Adenocarcinoma, large cell, non-small cell lung cancer	lung	human	NCI-H1581	CRL-5878
Adenocarcinoma, malignant, clear cell carcinoma	ovary	human	TOV-21G	CRL-11730†
Adenocarcinoma, malignant, endometrioid carcinoma	ovary	human	TOV-112D	CRL-11731†

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Tumor Cell Lines by Disease

Disease	Source	Species	Name	ATCC® No.
Adenocarcinoma, non-small cell lung cancer	lung	human	NCI-H23	CRL-5800
Adenocarcinoma, non-small cell lung cancer	lung	human	NCI-H522	CRL-5810
Adenocarcinoma, non-small cell lung cancer	lung	human	NCI-H1435	CRL-5870
Adenocarcinoma, non-small cell lung cancer	lung	human	NCI-H1563	CRL-5875
Adenocarcinoma, non-small cell lung cancer	lung	human	NCI-H1651	CRL-5884
Adenocarcinoma, non-small cell lung cancer	lung	human	NCI-H1734	CRL-5891
Adenocarcinoma, non-small cell lung cancer	lung	human	NCI-H1793	CRL-5896
Adenocarcinoma, non-small cell lung cancer	lung	human	NCI-H1838	CRL-5899
Adenocarcinoma, non-small cell lung cancer	lung	human	NCI-H1975	CRL-5908
Adenocarcinoma, non-small cell lung cancer	lung	human	NCI-H2073	CRL-5918
Adenocarcinoma, non-small cell lung cancer	lung	human	NCI-H2085	CRL-5921
Adenocarcinoma, non-small cell lung cancer	lung	human	NCI-H2228	CRL-5935
Adenocarcinoma, non-small cell lung cancer	lung	human	NCI-H2342	CRL-5941
Adenocarcinoma, non-small cell lung cancer	lung	human	NCI-H2347	CRL-5942
Adenocarcinoma, renal	kidney	mouse	RAG	CCL-142
Adenocarcinoma, renal cell	kidney	human	ACHN	CRL-1611
Adenocarcinoma, renal cell	kidney	human	786-O	CRL-1932
Adenocarcinoma, renal cell	kidney	human	769-P	CRL-1933
Adenocarcinoma	pancreas	human	Panc 10.05	CRL-2547
Adenocarcinoma, scirrhous	mammary gland	human	Hs 742.T	CRL-7482*
Adenocarcinoma, squamous cell carcinoma; mixed small cell lung cancer	lung	human	NCI-H2066	CRL-5917
Adenocarcinoma, squamous cell carcinoma; mixed small cell lung cancer	lung	human	NCI-H2286	CRL-5938
Adenocarcinoma, squamous cell, non-small cell lung cancer	lung	human	NCI-H1703	CRL-5889
Adenoma	lung	mouse	LA-4	CCL-196
Adenoma	pancreas, alpha cell	mouse, transgenic	αTC1 Clone 9	CRL-2350
Adenoma	pancreas, beta cell	mouse, transgenic	NIT-2	CRL-2364
Adenoma	pituitary, anterior	rat	RC-4B/C	CRL-1903
Adenomatosis, hereditary	skin	human	182-PF SK	CRL-1532
Adenomatosis, hereditary (Gardner's variant)	skin	human	166-ME SK	CRL-1533
Angiomyolipoma	kidney	human	SV7tert	CRL-2461
Astrocytoma	brain	human	CCF-STTG1	CRL-1718
Astrocytoma	brain	human	SW 1088	HTB-12
Astrocytoma	brain	human	SW 1783	HTB-13
Cancer	breast, nipple	human	HT 762.T	CRL-7789*
Cancer	connective tissue	dog	CF17.T	CRL-6219*
Cancer	connective tissue	dog	CF21.T	CRL-6220*
Cancer	connective tissue	dog	CF24.T	CRL-6221*
Cancer	connective tissue	mouse	MM36T(C)	CRL-6411*
Cancer	connective tissue	mouse	MM37T	CRL-6414*
Cancer	lung	human	Hs 573.T	CRL-7343*
Cancer	mammary gland	dog	CF33.MT	CRL-6227*

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Tumor Cell Lines by Disease

Disease	Source	Species	Name	ATCC® No.
Cancer	mammary gland	dog	CF34.Mg	CRL-6228*
Cancer	mammary gland	dog	CF35.Mg	CRL-6229*
Cancer	mammary gland	dog	CF41.Mg	CRL-6232*
Cancer	mammary gland	dog	CF45B.Mg	CRL-6237*
Cancer	mammary gland	human	Hs 190.T	CRL-7145*
Cancer	mammary gland	human	Hs 319.T	CRL-7236*
Cancer	mammary gland	human	Hs 329.T	CRL-7242*
Cancer	mammary gland	human	Hs 344.T	CRL-7246*
Cancer	mammary gland	human	Hs 350.T	CRL-7248*
Cancer	mammary gland	human	Hs 371.T	CRL-7256*
Cancer	mammary gland	human	Hs 748.T	CRL-7486*
Cancer	mammary gland	human	Hs 841.T	CRL-7574*
Cancer	mammary gland	human	Hs 849.T	CRL-7583*
Cancer	mammary gland	human	Hs 851.T	CRL-7584*
Cancer	mammary gland	human	Hs 861.T	CRL-7596*
Cancer	mammary gland	human	Hs 905.T	CRL-7652*
Cancer	mammary gland	human	Hs 479.T	CRL-7813*
Cancer	mammary gland	monkey, Rhesus	CMMT	CRL-6299*
Cancer	mammary gland	mouse	MM2MT	CRL-6373*
Cancer	mammary gland	mouse	MM2MTC	CRL-6374*
Cancer	mammary gland	mouse	MM2SCT	CRL-6375*
Cancer	mammary gland	mouse	MM5MTC	CRL-6378*
Cancer	mammary gland	mouse	MM5MTM	CRL-6379*
Cancer	mammary gland	mouse	MM5.1	CRL-6380*
Cancer	mammary gland	mouse	MM5/C1	CRL-6444*
Cancer	mammary gland	mouse	RIIIMT	CRL-6449*
Cancer	mammary gland	mouse	+/+ MGT	CRL-6468*
Cancer	mammary gland	mouse	MM5MT	CRL-6590*
Cancer	mammary gland	rat	Rn1T	CRL-6598*
Cancer	mammary gland	rat	Rn2T	CRL-6599*
Cancer	mixed connective and soft tissue	mouse	+/+ SCT	CRL-6469*
Cancer	prostate	rat	R-3327-AT-1	JHU-29
Cancer	prostate	rat	R3327-G	JHU-3
Cancer	prostate	rat	R-3327-AT-2.1	JHU-30
Cancer	prostate	rat	R-3327-AT-3.1	JHU-31
Cancer	prostate	rat	MAT-Lu	JHU-4
Cancer	unknown	mouse	CFZT(A)	CRL-6338*
Cancer	unknown	mouse	CFZT(B)	CRL-6339*
Cancer	unknown	mouse	MM14.OT	CRL-6384*
Cancer	unknown	mouse	MM43T	CRL-6418*
Cancer	unknown	mouse	MM15OT	CRL-6438*
Cancer, colorectal	colon	human	Hs 675.T	CRL-7400*
Cancer, non-small cell lung	lung	human	NCI-H2135	CRL-5926
Cancer, non-small cell lung	lung	human	NCI-H2172	CRL-5930
Cancer, non-small cell lung	lung	human	NCI-H2444	CRL-5945
Carcinoid	lung	human	NCI-H835	CRL-5843
Carcinoid	lung	human	UMC-11	CRL-5975
Carcinoid	lung, bronchus	human	NCI-H727	CRL-5815
Carcinoid, atypical	lung	human	NCI-H720	CRL-5838
Carcinoma	adrenal gland, cortex	human	NCI-H295R	CRL-2128
Carcinoma	bladder, urinary	human	Hs 195.T	CRL-7150*
Carcinoma	bladder, urinary	human	Hs 228.T	CRL-7193*
Carcinoma	bladder, urinary	human	Hs 172.T	CRL-7833*
Carcinoma	bladder, urinary	human	5637	HTB-9
Carcinoma	bladder, urinary	human	HT-1376	CRL-1472
Carcinoma	bladder, urinary	human	HT-1197	CRL-1473
Carcinoma	cervix	human	C-4 I	CRL-1594
Carcinoma	cervix	human	C-4 II	CRL-1595
Carcinoma	cervix	human	DoTc2 4510	CRL-7920*
Carcinoma	cervix	human	C-33 A	HTB-31

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Tumor Cell Lines by Disease

Disease	Source	Species	Name	ATCC® No.
Carcinoma	colon	mouse	CT26.WT	CRL-2638
Carcinoma	colon	mouse	CT26.CL25	CRL-2639
Carcinoma	embryo	mouse	NFPE	CRL-2069
Carcinoma	embryo	mouse	PFHR 9	CRL-2423
Carcinoma	embryo, yolk sac	rat	L2-RYC	CRL-2180
Carcinoma	embryonal stem cell	mouse	ES-E14TG2a	CRL-1821
Carcinoma	embryonal stem cell, germ line competent	mouse	ES-D3 GL	SCRC-1003
Carcinoma	kidney	human	A-498	HTB-44
Carcinoma	lung	human	A549	CCL-185
Carcinoma	lung	human	A-427	HTB-53
Carcinoma	mammary gland	human	Hs 540.T	CRL-7316*
Carcinoma	mammary gland	human	Hs 566(B).T	CRL-7336*
Carcinoma	mammary gland	human	Hs 605.T	CRL-7365*
Carcinoma	mammary gland	human	Hs 606	CRL-7368*
Carcinoma	mammary gland	human	BT-20	HTB-19
Carcinoma	mammary gland	mouse	CMH1a	CRL-8399†
Carcinoma	mammary gland	mouse	CSMαβ6C	CRL-8400†
Carcinoma	mammary gland	mouse	CSMαβ1H	CRL-8401†
Carcinoma	ovary	human	Hs 38.T	CRL-7826*
Carcinoma	ovary	human	Hs 571.T	CRL-7846*
Carcinoma	pancreas	human	MIA PaCa-2	CRL-1420
Carcinoma	pancreas	rat	DSL-6A/C1	CRL-2132
Carcinoma	pancreas	rat	DSL-6B/C2	CRL-2133
Carcinoma	prostate	human	22Rv1	CRL-2505
Carcinoma	salivary gland, submandibular	mouse	SCA-9 clone 15	CRL-1734
Carcinoma	stomach	human	Hs 740.T	CRL-7870*
Carcinoma	thyroid, medulla, C cell	mouse	MTC-M	CRL-1806
Carcinoma	unknown	mouse	Ehrlich-Lettre ascites, strain E	CCL-77
Carcinoma	unknown	rat	LLC-WRC 256	CCL-38
Carcinoma	uterus, endometrium	human	RL95-2	CRL-1671
Carcinoma	yolk sac, parietal endoderm	mouse	PYS-2	CRL-2745
Carcinoma, acinar cell	pancreas	mouse, transgenic	TGP49	CRL-2136
Carcinoma, acinar cell	pancreas	mouse, transgenic	TGP47	CRL-2141
Carcinoma, adenosquamous	lung	human	NCI-H596	HTB-178
Carcinoma, adrenocortical	adrenal gland, cortex	human	NCI-H295	CRL-10296†
Carcinoma, alveolar cell	lung	human	SW 1573	CRL-2170
Carcinoma, basal cell	skin	human	TE 354.T	CRL-7762*
Carcinoma, bronchioalveolar, non-small cell lung cancer	lung, bronchiole, alveolus	human	NCI-H358	CRL-5807
Carcinoma, classic small cell lung cancer	lung	human	NCI-H1688	CCL-257
Carcinoma, classic small cell lung cancer	lung	human	NCI-H1417	CRL-5869
Carcinoma, classic small cell lung cancer	lung	human	NCI-H1672	CRL-5886
Carcinoma, classic small cell lung cancer	lung	human	NCI-H1836	CRL-5898
Carcinoma, clear cell	kidney	human	Caki-2	HTB-47
Carcinoma, clear cell	ovary	human	ES-2	CRL-1978
Carcinoma, colorectal	cecum	human	SNU-C2B	CCL-250
Carcinoma, colorectal	cecum	human	SNU-C2A	CCL-250.1
Carcinoma, colorectal	cecum	human	LS513	CRL-2134
Carcinoma, colorectal	cecum	human	LS1034	CRL-2158
Carcinoma, colorectal	cecum	human	LS411N	CRL-2159
Carcinoma, colorectal	colon	human	HCT 116	CCL-247
Carcinoma, colorectal	colon	human	ATRFLOX	CRL-2780
Carcinoma, colorectal	rectum	human	Hs 722.T	CRL-7456*
Carcinoma, ductal	mammary gland	human	UACC-812	CRL-1897

* Part of the NBL collection; see page 12. † Patent item; see page 12.
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Tumor Cell Lines by Disease

Disease	Source	Species	Name	ATCC® No.
Carcinoma, ductal	mammary gland	human	HCC1954	CRL-2338
Carcinoma, ductal	mammary gland	human	Hs 574.T	CRL-7345*
Carcinoma, ductal	mammary gland	human	BT-483	HTB-121
Carcinoma, ductal	mammary gland	human	BT-549	HTB-122
Carcinoma, ductal	mammary gland	human	DU4475	HTB-123
Carcinoma, ductal	mammary gland	human	Hs 578T	HTB-126
Carcinoma, ductal	mammary gland	human	BT-474	HTB-20
Carcinoma, embryonal	testis	human	Cates-1B	HTB-104
Carcinoma, embryonal, testicular teratoma	testis	mouse	F9	CRL-1720
Carcinoma, epidermoid	epidermis	human	A-431	CRL-1555
Carcinoma, epidermoid	epidermis	human	A431NS	CRL-2592
Carcinoma, epidermoid	epidermis	human	A253	CRL-7902*
Carcinoma, epidermoid	lung	human	HLF-a	CCL-199
Carcinoma, epidermoid	salivary gland, submaxillary	human	A-253	HTB-41
Carcinoma, epithelioid	pancreas, duct	human	PANC-1	CRL-1469
Carcinoma, gastric	stomach	human	SNU-1	CRL-5971
Carcinoma, hepatocellular	liver	chicken	LMH	CRL-2117
Carcinoma, hepatocellular	liver	chicken	LMH/2A	CRL-2118
Carcinoma, hepatocellular	liver	human	C3A	CRL-10741†
Carcinoma, hepatocellular	liver	human	SNU-398	CRL-2233
Carcinoma, hepatocellular	liver	human	SNU-449	CRL-2234
Carcinoma, hepatocellular	liver	human	SNU-182	CRL-2235
Carcinoma, hepatocellular	liver	human	SNU-475	CRL-2236
Carcinoma, hepatocellular	liver	human	Hep 3B2.1-7	HB-8064
Carcinoma, hepatocellular	liver	human	Hep G2	HB-8065
Carcinoma, hepatocellular	liver	topminnow	PLHC-1	CRL-2406
Carcinoma, large cell, neuro-endocrine, non-small cell lung cancer	lung	human	NCI-H810	CRL-5816
Carcinoma, Lewis lung	lung	mouse	LL/2 (LLC1)	CRL-1642
Carcinoma, malignant	prostate	rat	AT3B-1	CRL-2375
Carcinoma, malignant	prostate	rat	MAT-Ly-Lu-B-2	CRL-2376
Carcinoma, mammary	breast	mouse	EMT6	CRL-2755
Carcinoma, medulla	thyroid	human	TT	CRL-1803
Carcinoma, medullary thyroid	thyroid	rat	6-23 (Clone 6)	CRL-1607
Carcinoma, mucoepidermoid pulmonary	lung	human	NCI-H292	CRL-1848
Carcinoma, non-small cell lung cancer	lung	human	NCI-H2126	CCL-256
Carcinoma, papilloma virus induced	unknown	rabbit	VX7	CRL-6504*
Carcinoma, pleomorphic hepatocellular	liver	human	SNU-387	CRL-2237
Carcinoma, pleomorphic hepatocellular	liver	human	SNU-423	CRL-2238
Carcinoma, polyploid	rectum	mouse	CMT-93	CCL-223
Carcinoma, primary acantholytic squamous cell	mammary gland	human	HCC1806	CRL-2335
Carcinoma, primary ductal	mammary gland	human	UACC-893	CRL-1902
Carcinoma, primary ductal	mammary gland	human	HCC38	CRL-2314
Carcinoma, primary ductal	mammary gland	human	HCC70	CRL-2315
Carcinoma, primary ductal	mammary gland	human	HCC202	CRL-2316
Carcinoma, primary ductal	mammary gland	human	HCC1143	CRL-2321
Carcinoma, primary ductal	mammary gland	human	HCC1187	CRL-2322
Carcinoma, primary ductal	mammary gland	human	HCC1395	CRL-2324
Carcinoma, primary ductal	mammary gland	human	HCC1419	CRL-2326
Carcinoma, primary ductal	mammary gland	human	HCC1500	CRL-2329
Carcinoma, primary ductal	mammary gland	human	HCC1599	CRL-2331
Carcinoma, primary ductal	mammary gland	human	HCC1937	CRL-2336
Carcinoma, primary ductal	mammary gland	human	HCC2157	CRL-2340

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Tumor Cell Lines by Disease

Disease	Source	Species	Name	ATCC® No.
Carcinoma, primary ductal	mammary gland	human	HCC2218	CRL-2343
Carcinoma, primary metaplastic	mammary gland	human	HCC1569	CRL-2330
Carcinoma, primary small cell	adrenal gland, cortex	human	SW-13	CCL-105
Carcinoma, probably lung anaplastic	unknown	human	Calu-6	HTB-56
Carcinoma, small cell lung cancer	lung	human	DMS 79	CRL-2049
Carcinoma, small cell lung cancer	lung	human	DMS 53	CRL-2062
Carcinoma, small cell lung cancer	lung	human	DMS 114	CRL-2066
Carcinoma, small cell lung cancer	lung	human	SW 1271	CRL-2177
Carcinoma, small cell lung cancer	lung	human	NCI-H2227	CRL-5934
Carcinoma, small cell lung cancer	lung	human	NCI-H1963	CRL-5982
Carcinoma, small cell lung cancer, large cell, variant	lung	human	SHP-77	CRL-2195
Carcinoma, small cell lung cancer, multidrug resistant	lung	human	H69AR	CRL-11351†
Carcinoma, squamous cell	cervix	human	SW756	CRL-10302†
Carcinoma, squamous cell	cervix	human	SiHa	HTB-35
Carcinoma, squamous cell	lung	human	NCI-H2170	CRL-5928
Carcinoma, squamous cell	lung	human	NCI-H520	HTB-182
Carcinoma, squamous cell	lung	human	SW 900	HTB-59
Carcinoma, squamous cell	lung	mouse	KLN 205	CRL-1453
Carcinoma, squamous cell	nasal	rat	FAT 7	CRL-2109
Carcinoma, squamous cell	pharynx	human	FaDu	HTB-43
Carcinoma, squamous cell	thyroid	human	SW579	HTB-107
Carcinoma, squamous cell	tongue	human	SCC-15	CRL-1623
Carcinoma, squamous cell	tongue	human	SCC-4	CRL-1624
Carcinoma, squamous cell	tongue	human	SCC-25	CRL-1628
Carcinoma, squamous cell	tongue	human	SCC-9	CRL-1629
Carcinoma, squamous cell	tongue	human	CAL 27	CRL-2095
Carcinoma, squamous cell	vulva	human	SW 954	HTB-117
Carcinoma, transitional cell	bladder, urinary	human	UM-UC-3	CRL-1749
Carcinoma, transitional cell	bladder, urinary	human	SW 780	CRL-2169
Carcinoma, transitional cell	bladder, urinary	human	J82	HTB-1
Carcinoma, transitional cell	bladder, urinary	human	SCaBER	HTB-3
Carcinoma, transitional cell	bladder, urinary	human	T24	HTB-4
Carcinoma, transitional cell	bladder, urinary	human	TCCSUP	HTB-5
Carcinoma, transitional cell	ureter	human	Hs 789.T	CRL-7886*
Carcinoma, transitional cell	urethra	human	Hs 769.T	CRL-7882*
Chondrosarcoma	bone	human	Hs 819.T	CRL-7891*
Chondrosarcoma	bone	human	SW 1353	HTB-94
Choriocarcinoma	placenta	human	BeWo	CCL-98
Choriocarcinoma	placenta	human	JAR	HTB-144
Choriocarcinoma	placenta	human	JEG-3	HTB-36
Dermatofibrosarcoma	skin	human	Hs 357.T	CRL-7252*
Dermatofibrosarcoma	skin	human	Hs 941.T	CRL-7692*
Dermatofibrosarcoma protuberans	skin	human	Hs 295.T	CRL-7233*
Dermatofibrosarcoma protuberans	skin	human	Hs 63.T	CRL-7043*
Endothelioma	brain, cerebral cortex; transformed	mouse	bEnd.3	CRL-2299
Erythroleukemia	bone marrow	cat	F25	CRL-6566*
Erythroleukemia	bone marrow, erythroblast	human	TF-1	CRL-2003
Erythroleukemia	bone marrow, erythroblast	human	TF-1a	CRL-2451
Erythroleukemia	bone marrow, erythroblast	human	TF-1.CN5a.1	CRL-2512
Erythroleukemia	bone marrow, erythroblast	human	HEL 92.1.7	TIB-180
Fibroma	connective tissue	gerbil, Mongolian	IMR-33	CCL-146
Fibromatosis	mixed connective and soft tissue	human	TE 115.T	CRL-7744*
Fibrosarcoma	bladder (adjacent)	mouse	MM45T.BI	CRL-6420*

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Tumor Cell Lines by Disease

Disease	Source	Species	Name	ATCC® No.
Fibrosarcoma	connective tissue	cat	FC77.T	CRL-6105*
Fibrosarcoma	connective tissue	cat	FC81.T	CRL-6108*
Fibrosarcoma	connective tissue	cat	FC94.T	CRL-6113*
Fibrosarcoma	connective tissue	human	HT-1080	CCL-121
Fibrosarcoma	connective tissue	human	Hs 778(A).T	CRL-7508*
Fibrosarcoma	connective tissue	human	Hs 778(B).T	CRL-7509*
Fibrosarcoma	connective tissue	human	Hs 15.T	CRL-7824*
Fibrosarcoma	connective tissue	human	SW 684	HTB-91
Fibrosarcoma	connective tissue	mouse	$HSDM_1C_1$	CCL-148
Fibrosarcoma	connective tissue	mouse	MM47T	CRL-6424*
Fibrosarcoma	liver (adjacent)	mouse	MM45T.Li	CRL-6421*
Fibrosarcoma	mixed connective and soft tissue	human	Hs 93.T	CRL-7062*
Fibrosarcoma	spleen	cat	FC81.Sp	CRL-6107*
Fibrosarcoma	spleen	cat	FC83.Sp	CRL-6110*
Fibrosarcoma	spleen	mouse	MM45T.Sp	CRL-6422*
Fibrosarcoma	spleen	mouse	MM52.Sp	CRL-6428*
Fibrosarcoma	spleen	mouse	MM53.Sp	CRL-6430*
Fibrosarcoma	thymus	cat	FC81.Thy	CRL-6109*
Fibrosarcoma	unknown	human	Hs 868.T	CRL-7604*
Fibrosarcoma	unknown	mouse	WEHI-13VAR	CRL-2148
Fibrosarcoma	unknown	mouse	MM46T	CRL-6423*
Fibrosarcoma	unknown	mouse	MM48T	CRL-6425*
Fibrosarcoma	unknown	mouse	MM49T	CRL-6426*
Fibrosarcoma	unknown	mouse	MM52.T	CRL-6429*
Fibrosarcoma	unknown	mouse	Sal/N	CRL-2544
Fibrosarcoma	unknown	mouse	TM-7	CRL-2798
Fibrosarcoma	unknown	mouse	MC17-51	CRL-2799
Fibrosarcoma	unknown	mouse	MN-11	CRL-2800
Fibrosarcoma	unknown	mouse	TM-34	CRL-2801
Fibrosarcoma	unknown	mouse	MiF-6	CRL-2802
Fibrosarcoma	unknown	mouse	TM-28	CRL-2803
Fibrosarcoma	unknown	mouse	M-7	CRL-2804
Fibrosarcoma	unknown	mouse	MT-6	CRL-2805
Fibrosarcoma	unknown	quail, Japanese	QT6	CRL-1708
Fibrosarcoma, chemically induced	muscle	quail, Japanese	QM7	CRL-1962
Fibrosarcoma, malignant, dibenzanthracene induced	ascites	mouse	Sal	CRL-2543
Fibrosarcoma, methylcholanthracene induced	unknown	mouse	WEHI 164	CRL-1751
Fibrosarcoma, methylcholanthrocene induced	unknown	mouse	MC57G	CRL-2295
Glioblastoma	brain	human	A172	CRL-1620
Glioblastoma	brain	human	U-138 MG	HTB-16
Glioblastoma	brain, glial cell	human	DBTRG-05MG	CRL-2020
Glioblastoma	brain	human	LN-18	CRL-2610
Glioblastoma	brain	human	LN-229	CRL-2611
Glioblastoma, astrocytoma	brain	human	U-87 MG	HTB-14
Glioblastoma, astrocytoma	brain	human	U-118 MG	HTB-15
Glioblastoma, malignant	brain, glial cell	human	M059K	CRL-2365
Glioblastoma, malignant	brain, glial cell	human	M059J	CRL-2366
Glioblastoma, multiforme	brain	human	T98G	CRL-1690
Glioblastoma, neuroblastoma	brain, glial cell, neuron	mouse/rat hybrid	NG108-15	HB-12317†
Glioblastoma, p53 expression	brain	human	LNZTA3WT4	CRL-11543†
Glioblastoma, p53 expression	brain	human	LNZTA3WT11	CRL-11544†
Glioma	brain	human	Hs 683	HTB-138
Glioma	brain	rat	C_6	CCL-107
Glioma, undifferentiated malignant	brain, fetal	rat	F98	CRL-2397
Glioma, undifferentiated malignant	brain, fetal	rat	RG2	CRL-2433
Gliosarcoma, expresses beta galactosidase	brain	rat	C6/LacZ	CRL-2199

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Tumor Cell Lines by Disease

Disease	Source	Species	Name	ATCC® No.
Gliosarcoma, expresses beta galactosidase	brain	rat	9L/lacZ	CRL-2200
Gliosarcoma, expresses beta galactosidase	brain	rat	C6/lacZ7	CRL-2303
Glomangioma	kidney, glomus	human	glomotel	CRL-2597
Granuloma, eosinophilic	bone	human	Hs 454.T	CRL-7802*
Granuloma, noncaseating	lymph node	human	Hs 697.Ln	CRL-7434*
Granuloma, periostitis	bone	human	Hs 709.T	CRL-7453*
Hemangioendothelioma	unknown	mouse	EOMA	CRL-2586
Hemangioendothelioma; expresses GFP	unknown	mouse	EOMA-GFP	CRL-2587
Hepatoma	liver	mouse	Hepa 1-6	CRL-1830
Hepatoma	liver	mouse	Hepa-1c1c7	CRL-2026
Hepatoma	liver	mouse	BpRcl	CRL-2217
Hepatoma	liver	mouse	tao BpRcl	CRL-2218
Hepatoma	liver	rat	MH_1C_1	CCL-144
Hepatoma	liver	rat	H-4-II-E	CRL-1548
Hepatoma	liver	rat	H4TG	CRL-1578
Hepatoma	liver	rat	H4-II-E-C3	CRL-1600
Hepatoma	liver	trout, rainbow	RTH-149	CRL-1710
Hepatoma	liver	woodchuck, Eastern	WCH-17	CRL-2082
Hepatoma	liver, Alexander cells	human	PLC/PRF/5	CRL-8024†
Hepatoma; deficient in aryl hydrocarbon hydoxylase activity	liver	mouse	c37 (B7!Fi1)	CRL-2711
Hepatoma; deficient in aryl hydrocarbon hydoxylase activity	liver	mouse	c1 (B6NLxv1c2)	CRL-2716
Hepatoma; lacks functional aryl hydrocarbon receptor nuclear translocator protein	liver	mouse	c4 (B13NBii1)	CRL-2717
Hepatoma, Morris Hepatoma 7777	liver	rat	McA-RH7777	CRL-1601
Hepatoma, Morris Hepatoma 8994	liver	rat	McA-RH8994	CRL-1602
Hepatoma, Novikoff Hepatoma	liver	rat	N1-S1 Fudr	CRL-1603
Hepatoma, Novikoff Hepatoma	liver	rat	N1-S1	CRL-1604
Hepatoma; reduced levels of aryl AHR mRNA and protein	liver	mouse	c12 (B15ECiii2)	CRL-2710
Hepatoma; reduced levels of aryl hydrocarbon hydroxylase	liver	mouse	vT{2}	CRL-2712
Hepatoma; reduced levels of aryl hydrocarbon hydroxylase	liver	mouse	c35 (B16GBi1c3)	CRL-2715
Histiocytoma	connective tissue	human	Hs 856.T	CRL-7593*
Histiocytoma, fibrous, malignant	unknown	mouse	p53NiS1	CRL-2619
Histiocytosis, chronically infected with *Ehrlichia canis*	macrophage	dog	DH82ECOK	CRL-10390†
Histiocytosis, malignant	macrophage	dog	DH82	CRL-10389†
Hodgkin's disease	lymphoblast, peripheral blood	human	RPMI 6666	CCL-113
Hypernephroma	kidney	human	SW 156	CRL-2175
Insulinoma	pancreas, beta cell	mouse, transgenic	β-TC-6	CRL-11506†
Insulinoma	pancreas, islet of Langerhans	mouse, transgenic	NIT-1	CRL-2055
Insulinoma; produces insulin and and L-dopa decarboxylase but not somatostatin	pancreas, islet of Langerhans	rat	RIN-m5F	CRL-11605†
Insulinoma; produces insulin and L-dopa-decarboxylase but not somatostatin	pancreas, islet of Langerhans	rat	RIN-5F	CRL-2058
Insulinoma; produces insulin and somatostatin	pancreas, islet of Langerhans	rat	RIN-m	CRL-2057
Insulinoma; produce somatostatin and L-dopa-decarboxylase but not insulin	pancreas, islet of Langerhan	rat	RIN-14B	CRL-2059

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Tumor Cell Lines by Disease

Disease	Source	Species	Name	ATCC® No.
Interscapular tumor	possibly basal cell	bat, mouse-eared	Mvi/It	CRL-6012*
Keratoacanthoma	skin	human	Hs 892.T	CRL-7630*
Keratoacanthoma, malignant acanthocytosis	skin	human	Hs 898.T	CRL-7641*
Leiomyoblastoma, renal	kidney	human	G-402	CRL-1440
Leiomyosarcoma	muscle	human	TE 149.T	CRL-7751*
Leiomyosarcoma	connective tissue	human	Hs 5.T	CRL-7822*
Leiomyosarcoma	smooth muscle, ductus deferens	hamster, Syrian golden	DDT_1 MF-2	CRL-1701
Leiomyosarcoma	uterus	human	SK-UT-1	HTB-114
Leiomyosarcoma	uterus, endometrium	human	SK-UT-1B	HTB-115
Leiomyosarcoma	vulva	human	SK-LMS-1	HTB-88
Leukemia	B lymphocyte	mouse	CW13.20-3B3 (clone of BCL 1)	CRL-1669
Leukemia	basophil, peripheral blood	rat	RBL-1	CRL-1378
Leukemia	basophil, peripheral blood	rat	RBL-2H3	CRL-2256
Leukemia	erythroblast, spleen	mouse	BB88	TIB-55
Leukemia	erythroblast, spleen	mouse	D1B	TIB-56
Leukemia	lymph node	bovine	2FLB.Ln	CRL-6045*
Leukemia	myelomonocyte, macrophage-like	mouse	WEHI-3	TIB-68
Leukemia	spleen	mouse	T27A	TIB-57
Leukemia	spleen	mouse	D2N	TIB-58
Leukemia	spleen	mouse	BC16A	TIB-59
Leukemia	spleen	mouse	BC3A	TIB-60
Leukemia, acute lymphoblastic	B lymphoblast	human	SUP-B15	CRL-1929
Leukemia, acute lymphoblastic	B lymphoblast, peripheral blood	human	CCRF-SB	CCL-120
Leukemia, acute lymphoblastic	B lymphoblast, peripheral blood	human	8E5	CRL-8993†
Leukemia, acute lymphoblastic	bone marrow, myeloblast	human	KG-1	CCL-246
Leukemia, acute lymphoblastic	bone marrow, myeloblast	human	KG-1	CRL-8031†
Leukemia, acute lymphoblastic	bone marrow, promyeloblast	human	KG-1a	CCL-246.1
Leukemia, acute lymphoblastic	T lymphoblast	human	TALL-104	CRL-11386†
Leukemia, acute lymphoblastic	T lymphoblast	human	MOLT-4	CRL-1582
Leukemia, acute lymphoblastic	T lymphoblast, peripheral blood	human	CCRF-CEM	CCL-119
Leukemia, acute lymphoblastic	T lymphoblast, peripheral blood	human	CCRF-HSB-2	CCL-120.1
Leukemia, acute lymphoblastic	T lymphoblast, peripheral blood	human	MOLT-3	CRL-1552
Leukemia, acute lymphoblastic	T lymphoblast, peripheral blood	human	CEM/C2	CRL-2264
Leukemia, acute lymphoblastic	T lymphoblast, peripheral blood	human	CEM/C1	CRL-2265
Leukemia, acute lymphoblastic t(16;20) translocation	T lymphocyte, peripheral blood	human	Loucy	CRL-2629
Leukemia, acute lymphoblastic, t(4;11) translocation	bone marrow	human	RS4;11	CRL-1873
Leukemia, acute lymphocytic (non-T, non-B)	unknown	human	Reh	CRL-8286†
Leukemia, acute monocytic	monocyte	human	THP-1	TIB-202
Leukemia, acute monocytic	monocyte, peripheral blood	human	AML-193	CRL-9589†
Leukemia, acute myeloblastic	peripheral blood	human	Kasumi-1	CRL-2724
Leukemia, acute myeloblastic	peripheral blood	human	Kasumi-3	CRL-2725
Leukemia, acute myelogenous	peripheral blood, B lymphoblast	human	BDCM	CRL-2740

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Tumor Cell Lines by Disease

Disease	Source	Species	Name	ATCC® No.
Leukemia, acute myeloid	unknown	mouse	C1498	TIB-49
Leukemia, acute myeloid	peripheral blood	human	AML14.3D10/CCCKR3 Clone 16	CRL-12079†
Leukemia, acute myeloid, subtype M2	peripheral blood	human	Kasumi-6	CRL-2775
Leukemia, acute promyelocytic	promyeloblast, peripheral blood	human	HL-60	CCL-240
Leukemia, acute promyelocytic	promyeloblast, peripheral blood	human	Clone 15 HL-60	CRL-1964
Leukemia, acute promyelocytic	promyeloblast, peripheral blood	human	HL-60/MX2	CRL-2257
Leukemia, acute promyelocytic	promyeloblast, peripheral blood	human	HL-60/MX1	CRL-2258
Leukemia, acute T cell	T lymphocyte	human	J.CaM1.6	CRL-2063
Leukemia, acute T cell	T lymphocyte	human	Jurkat, Clone E6-1	TIB-152
Leukemia, acute T cell	T lymphocyte	human	J.RT3-T3.5	TIB-153
Leukemia, acute T cell, CD4 negative	T lymphoblast	human	D1.1	CRL-10915†
Leukemia, acute T cell, CD45 deficient	T lymphocyte	human	J45.01	CRL-1990
Leukemia, biphenotypic B myelomonocytic	peripheral blood	human	MV-4-11	CRL-9591†
Leukemia, chronic myeloblastic	peripheral blood	human	Kasumi-4	CRL-2726
Leukemia, chronic myelogenous	basophil, peripheral blood	human	KU812	CRL-2099
Leukemia, chronic myelogenous	basophil, peripheral blood	human	KU812E	CRL-2100
Leukemia, chronic myelogenous	basophil, peripheral blood	human	KU812F	CRL-2101
Leukemia, chronic myelogenous	bone marrow, megakaryoblast	human	MEG-01	CRL-2021
Leukemia, hairy cell	B lymphoblast, peripheral blood	human	Mo-B	CCL-245
Leukemia, hairy cell	T lymphocyte	human	Mo	CRL-8066†
Leukemia, lymphoblastic	T lymphoblast	human	SUP-T1	CRL-1942
Leukemia, lymphocytic	unknown	mouse	L1210	CCL-219
Leukemia, lymphoma	B lymphocyte	mouse	BCL_1 clone $5B_1b$	TIB-197
Leukemia, lymphoma	pre-B lymphoblast	human	JM1	CRL-10423†
Leukemia, myeloid	myeloblast	mouse	M1	TIB-192
Leukemia, myeloid, virus induced	peripheral blood	mouse	M-NFS-60	CRL-1838
Leukemia, myelomonoblastic	monoblast, peripheral blood	human	GDM-1	CRL-2627
Leukemia, myelomonocytic	lymphoblast	human	CESS	TIB-190
Leukemia, plasma cell	B lymphoblast, peripheral blood	human	ARH-77	CRL-1621
Liposarcoma	connective tissue	human	SW 872	HTB-92
Lymphogranulomatosis	lymph node	human	Hs 268.T	CRL-7218*
Lymphoma	B lymphoblast	human	1A2	CRL-8119†
Lymphoma	B lymphocyte	monkey, Rhesus	LCL 8664	CRL-1805†
Lymphoma	B lymphocyte	mouse	WEHI-231	CRL-1702
Lymphoma	B lymphocyte	mouse	WEHI-279	CRL-1704
Lymphoma	B lymphocyte	mouse	2PK-3	TIB-203
Lymphoma	B lymphocyte	mouse	CH1	TIB-221
Lymphoma	B lymphocyte, spleen	mouse	RAW 8.1	TIB-50
Lymphoma	bursa	chicken	DT40	CRL-2111
Lymphoma	bursa	chicken	DT95	CRL-2112
Lymphoma	lymph node	human	Hs 313.T	CRL-7235*
Lymphoma	lymph node	human	Hs 777.T	CRL-7507*
Lymphoma	lymph node, cervical	human	Hs 602	HTB-142
Lymphoma	lymph node, submandibular	cat	F_1B	CRL-6168*
Lymphoma	lymphoblast	cat	FL74-UCD-1	CRL-8012†
Lymphoma	monocyte/macrophage	mouse	$P388D_1$	CCL-46
Lymphoma	monocyte/macrophage	mouse	NCTC 3749	CCL-46.1

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Tumor Cell Lines by Disease

Disease	Source	Species	Name	ATCC® No.
Lymphoma	monocyte/macrophage	mouse	PU5-1.8 (PU5-1R)	TIB-61
Lymphoma	monocyte/macrophage	mouse	P388D, (IL-1)	TIB-63
Lymphoma	pre-B lymphoblast	mouse	NFS-5 C-1	CRL-1693
Lymphoma	pre-B lymphoblast	mouse	NFS-25 C-3	CRL-1695
Lymphoma	pro-B lymphoblast	mouse	NFS-70 C-10	CRL-1694
Lymphoma	spleen	cat	FC16.Sp	CRL-6174*
Lymphoma	spleen	mouse	LBRM-33-1A5	CRL-8079†
Lymphoma	spleen	mouse	RAW 309F.1.1	TIB-51
Lymphoma	T lymphocyte	human	H9/HTLV-IIIB	CRL-8543†
Lymphoma	T lymphocyte	mouse	E.G7-OVA	CRL-2113
Lymphoma	T lymphocyte	mouse	TK-1	CRL-2396
Lymphoma	T lymphocyte	mouse	S1A(Thy-1⁻b)	TIB-231
Lymphoma	T lymphocyte	mouse	BW5147(T200⁻a)5.2	TIB-233
Lymphoma	T lymphocyte	mouse	S1A.TB.4.8.2	TIB-27
Lymphoma	T lymphocyte	mouse	S49.1	TIB-28
Lymphoma	T lymphocyte	mouse	S49.1H.1AG.6/2	TIB-29
Lymphoma	T lymphocyte	mouse	S49.1TB.2	TIB-30
Lymphoma	T lymphocyte	mouse	S49.1TB.4 DEX R.63	TIB-33
Lymphoma	T lymphocyte	mouse	S49.1G.3	TIB-34
Lymphoma	T lymphocyte	mouse	S49.1G.3 PHA.100/0	TIB-35
Lymphoma	T lymphocyte	mouse	S49 (Thy-1-a)	TIB-36
Lymphoma	T lymphocyte	mouse	TIMI.4G.1.3	TIB-38
Lymphoma	T lymphocyte	mouse	EL4	TIB-39
Lymphoma	T lymphocyte	mouse	EL4.BU.1.OUA'.1.1	TIB-41
Lymphoma	T lymphocyte, B lymphocyte, thymus	mouse	WEHI 22.1	TIB-54
Lymphoma	T lymphocyte, cutaneous	human	HuT 78	TIB-161
Lymphoma	T lymphocyte, thymus	mouse	BW5147.3(Thy-1⁻e).10	TIB-234
Lymphoma	T lymphocyte, thymus	mouse	R1.1	TIB-42
Lymphoma	T lymphocyte, thymus	mouse	R1E/TL8x.1	TIB-43
Lymphoma	T lymphocyte, thymus	mouse	R1.G1	TIB-44
Lymphoma	T lymphocyte, thymus	mouse	R1E/TL8x.1.G1.OUA'.1	TIB-45
Lymphoma	T lymphocyte, thymus	mouse	BW5147.3	TIB-47
Lymphoma	T lymphocyte, thymus	mouse	WEHI 7.1	TIB-53
Lymphoma	thymus	mouse	L5178-R (LY-R)	CRL-1722
Lymphoma	thymus	mouse	L5178-S (LY-S)	CRL-1723
Lymphoma	thymus	mouse	EL4.IL-2	TIB-181
Lymphoma	thymus	mouse	TIMI.4	TIB-37
Lymphoma	unknown	cat	FeLV-3281	CRL-9116†
Lymphoma	unknown	human	HT 1417	CRL-7797*
Lymphoma	unknown	mouse	L5178Y TK+/- (clone 3.7.2C)	CRL-9518†
Lymphoma	unknown	mouse	WR19L	TIB-52
Lymphoma, AMLV-transformed	pre-B lymphoblast	mouse	ABE-8.1/2	TIB-205
Lymphoma, B cell	peritoneal effusion (metastatic site: peritoneal cavity)	human	JSC-1	CRL-2769
Lymphoma, body cavity based	B lymphoblast, peripheral blood	human	BCP-1	CRL-2294
Lymphoma, Burkitt's	ascites, B lymphocyte	human	2B8	CRL-12569†
Lymphoma, Burkitt's	B lymphoblast,	human	Daudi	CCL-213
Lymphoma, Burkitt's	B lymphoblast, peripheral blood	human	NC-37	CCL-214
Lymphoma, Burkitt's	B lymphocyte	human	EB-3	CCL-85
Lymphoma, Burkitt's	B lymphocyte	human	Raji	CCL-86
Lymphoma, Burkitt's	B lymphocyte	human	Jiyoye	CCL-87
Lymphoma, Burkitt's	B lymphocyte	human	NAMALWA	CRL-1432
Lymphoma, Burkitt's	B lymphocyte	human	HS-Sultan	CRL-1484
Lymphoma, Burkitt's	B lymphocyte	human	CA46	CRL-1648
Lymphoma, Burkitt's	B lymphocyte	human	GA-10	CRL-2392
Lymphoma, Burkitt's	B lymphocyte	human	GA-10 (Clone 4)	CRL-2393
Lymphoma, Burkitt's	B lymphocyte	human	GA-10 (Clone 20)	CRL-2394

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Tumor Cell Lines by Disease

Disease	Source	Species	Name	ATCC® No.
Lymphoma, Burkitt's	kidney, B cell	human	20B8	CRL-12582†
Lymphoma, Burkitt's	kidney, B cell	human	HKB-11	CRL-12568†
Lymphoma, Burkitt's	kidney, peripheral blood, somatic cell hybrid	human	1G2	CRL-13005†
Lymphoma, Burkitt's	lymph node	human	2F7	CRL-10237†
Lymphoma, Burkitt's	upper maxilla	human	EB1	HTB-60
Lymphoma, Burkitt's (American)	B lymphocyte	human	Ramos (RA 1)	CRL-1596
Lymphoma, Burkitt's (American)	B lymphocyte	human	Ramos.2G6.4C10	CRL-1923
Lymphoma, cutaneous	T lymphocyte, peripheral blood	human	H9	HTB-176
Lymphoma, cutaneous T cell	T lymphocyte, peripheral blood	human	HH	CRL-2105
Lymphoma, cutaneous T cell, mycosis fungoides	T lymphocyte, peripheral blood	human	MJ	CRL-8294†
Lymphoma, diffuse large cell, non-Hodgkin's B cell	B lymphocyte, peripheral blood	human	Toledo	CRL-2631
Lymphoma, EBV and KSHV positive	B lymphoblast	human	BC-1	CRL-2230
Lymphoma, EBV and KSHV positive	B lymphoblast	human	BC-2	CRL-2231
Lymphoma, histiocytic	macrophage	human	U-937	CRL-1593.2
Lymphoma, histiocytic; neomycin resistant	macrophage	human	TUR	CRL-2367
Lymphoma, Hodgkin's disease	lymph node	human	Hs 604.T	CRL-7362*
Lymphoma, Hodgkin's disease	lymph node	human	Hs 751.T	CRL-7488*
Lymphoma, Hodgkin's disease	lymph node	human	Hs 445	HTB-146
Lymphoma, Hodgkin's disease	lymph node, spleen	human	Hs 611.T	CRL-7373*
Lymphoma, Hodgkin's disease	lymph node, thymus	human	Hs 616.T	CRL-7378*
Lymphoma, KSHV positive	B lymphoblast	human	BC-3	CRL-2277
Lymphoma, large cell	B lymphoblast	human	DB	CRL-2289
Lymphoma, lymphocytic	lymph node	human	Hs 505.T	CRL-7306*
Lymphoma, lymphocytic	lymph node	human	Hs 491.T	CRL-7818*
Lymphoma, lymphocytic	spleen	human	Hs 518.T	CRL-7313*
Lymphoma, methylnitrosourea induced	pre-B lymphoblast	mouse	70Z/3	TIB-158
Lymphoma, Mo-MuLV induced	unknown	mouse	YAC-1	TIB-160
Lymphoma, mycosis fungoides	T lymphocyte, cutaneous	human	HuT 102	TIB-162
Lymphoma, possible Burkitt's	lymph node	human	TE 161.T	CRL-7753*
Lymphoma, radiation induced	T lymphocyte	mouse	LBRM TG6	CRL-1778
Lymphoma, radiation induced	T lymphocyte	mouse	LBRM-33 clone 4A2	TIB-155
Lymphosarcoma	B lymphocyte	bovine	BL3.1	CRL-2306
Lymphosarcoma	bone marrow	bovine	LB9.Bm	CRL-6053*
Lymphosarcoma	bone marrow	bovine	LB10.Bm	CRL-6060*
Lymphosarcoma	lymph node	human	TE 175.T	CRL-7755*
Lymphosarcoma	mixed spleen, thymus, and bone marrow	bovine	LB9.Sp/Thy/Bm	CRL-6052*
Lymphosarcoma	spleen	bovine	LB9.Sp	CRL-6058*
Lymphosarcoma	spleen	bovine	LB10.Sp	CRL-6063*
Lymphosarcoma	spleen	bovine	LB11.Sp	CRL-6067*
Lymphosarcoma	thymus	bovine	LB9.Thy	CRL-6059*
Lymphosarcoma	thymus	bovine	LB10.Thy	CRL-6064*
Lymphosarcoma	thymus	bovine	LB11.Thy	CRL-6068*
Lymphosarcoma	unknown	mouse	MB III (de Bruyn-Gey)	CCL-32
Lymphosarcoma, leukemia	B lymphocyte	bovine	BL-3	CRL-8037†
Mastocytoma	mast cell	mouse	P815	TIB-64
Medulloblastoma	brain, cerebellum	human	D341 Med	HTB-187
Medulloblastoma, desmoplastic cerebellar	brain, cerebellum	human	Daoy	HTB-186
Melanoma	skin	human	WM-115	CRL-1675
Melanoma	skin	human	Hs 600.T	CRL-7360*
Melanoma	skin	human	Hs 688(A).T	CRL-7425*
Melanoma	skin	human	Hs 839.T	CRL-7572*
Melanoma	skin	human	Hs 852.T	CRL-7585*

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Tumor Cell Lines by Disease

Disease	Source	Species	Name	ATCC® No.
Melanoma	skin	human	Hs 906(A).T	CRL-7653*
Melanoma	skin	human	Hs 906(B).T	CRL-7654*
Melanoma	skin	human	Hs 908.Sk	CRL-7658*
Melanoma	skin	human	Hs 936.T	CRL-7686*
Melanoma	skin	human	Hs 936.T(C1)	CRL-7687*
Melanoma	skin	human	Hs 939.T	CRL-7690*
Melanoma	skin	human	A101D	CRL-7898*
Melanoma	skin	human	CHL-1	CRL-9446†
Melanoma	skin	human	HMCB (Human Melanoma Cell Bowles)	CRL-9607†
Melanoma	skin	mouse	B16-F0	CRL-6322*
Melanoma	skin	mouse	B16-F1	CRL-6323*
Melanoma	skin	mouse	B16-F10	CRL-6475*
Melanoma	skin, melanocyte	mouse	Clone M-3	CCL-53.1
Melanoma, amelanotic	skin	human	C32TG	CRL-1579
Melanoma, amelanotic	skin	human	C32	CRL-1585
Melanoma, malignant	connective tissue	human	Hs 934.T	CRL-7684*
Melanoma, malignant	connective tissue	human	Hs 935.T	CRL-7685*
Melanoma, malignant	skin	human	G-361	CRL-1424
Melanoma, malignant	skin	human	A-375	CRL-1619
Melanoma, malignant	skin	human	A375.S2	CRL-1872
Melanoma, malignant	skin	human	COLO 829	CRL-1974
Melanoma, malignant	skin	human	Hs 940.T	CRL-7691*
Melanoma, malignant	skin	human	HT-144	HTB-63
Melanoma, malignant	skin	human	Malme-3M	HTB-64
Melanoma, malignant	skin	human	RPMI-7951	HTB-66
Melanoma, malignant	skin	human	SK-MEL-5	HTB-70
Melanoma, malignant	skin	human	SK-MEL-24	HTB-71
Melanoma, malignant	skin	human	SK-MEL-28	HTB-72
Melanoma, malignant	skin	human	SK-MEL-31	HTB-73
Melanoma, melanotic	skin	hamster, Syrian golden	RPMI 1846	CCL-49
Melanoma, nodular, in vertical growth phase	skin, melanocyte	human	WM278	CRL-2809
Melanoma, nodular, in vertical growth phase	skin, melanocyte; from lung metastases in mice	human	451Lu	CRL-2813
Melanoma, primary superficial, in radial growth phase/vertical growth phase	skin	human	WM1552C	CRL-2808
Melanoma, primary superficial, in radial growth phase/vertical growth phase	skin, melanocyte	human	WM35	CRL-2807
Melanoma, primary superficial, in vertical growth phase	skin, melanocyte	human	WM793B	CRL-2806
Melanoma, primary superficial, in vertical growth phase	skin, melanocyte; from lung metastases in mice	human	1205Lu	CRL-2812
Melanoma, primary, in vertical growth phase	skin, melanocyte	human	WM39	CRL-2811
Melanoma, transfected to express filamin-1	skin	human	A7	CRL-2500
Neuroblastoma	brain	human	CHP-212	CRL-2273
Neuroblastoma	brain, neuroblast	human	IMR-32	CCL-127
Neuroblastoma	brain, neuroblast	mouse	Neuro-2a	CCL-131
Neuroblastoma	brain, neuroblast	mouse	NB41A3	CCL-147
Neuroblastoma	brain, neuroblast	mouse	N1E-115	CRL-2263
Neuroblastoma	central nervous system; nitrosoethylurea-induced	rat	B35	CRL-2754
Neuroglioma	brain	human	H4	HTB-148
Osteoma, benign osteoid	bone	human	Hs 900.T	CRL-7646*
Osteoma, benign osteoid	bone	human	Hs 903.T	CRL-7649*
Osteoma, benign osteoid	bone	human	Hs 919.T	CRL-7672*
Osteosarcoma	bone	dog	D17	CRL-6248*
Osteosarcoma	bone	dog	D22	CRL-6250*
Osteosarcoma	bone	dog	D17	CRL-8468†
Osteosarcoma	bone	human	143.98.2	CRL-11226†
Osteosarcoma	bone	human	G-292, clone A141B1	CRL-1423

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Tumor Cell Lines by Disease

Disease	Source	Species	Name	ATCC® No.
Osteosarcoma	bone	human	MG-63	CRL-1427
Osteosarcoma	bone	human	HOS	CRL-1543
Osteosarcoma	bone	human	KHOS/NP (R-970-5)	CRL-1544
Osteosarcoma	bone	human	KHOS-240S	CRL-1545
Osteosarcoma	bone	human	KHOS-321H	CRL-1546
Osteosarcoma	bone	human	MNNG/HOS (Cl #5)	CRL-1547
Osteosarcoma	bone	human	Hs 3.T	CRL-7005*
Osteosarcoma	bone	human	Hs 39.T	CRL-7023*
Osteosarcoma	bone	human	Hs 184.T	CRL-7134*
Osteosarcoma	bone	human	Hs 188.T	CRL-7140*
Osteosarcoma	bone	human	Hs 387.T	CRL-7263*
Osteosarcoma	bone	human	Hs 704.T	CRL-7444*
Osteosarcoma	bone	human	Hs 707(A).T	CRL-7448*
Osteosarcoma	bone	human	Hs 735.T	CRL-7471*
Osteosarcoma	bone	human	Hs 755(B).T	CRL-7489*
Osteosarcoma	bone	human	Hs 781.T	CRL-7511*
Osteosarcoma	bone	human	Hs 792(B).T	CRL-7521*
Osteosarcoma	bone	human	Hs 805.T	CRL-7537*
Osteosarcoma	bone	human	Hs 811.T	CRL-7543*
Osteosarcoma	bone	human	Hs 866.T	CRL-7602*
Osteosarcoma	bone	human	Hs 870.T	CRL-7606*
Osteosarcoma	bone	human	Hs 871.T	CRL-7609*
Osteosarcoma	bone	human	Hs 889.T	CRL-7626*
Osteosarcoma	bone	human	Hs 890.T	CRL-7628*
Osteosarcoma	bone	human	Murphy	CRL-7722*
Osteosarcoma	bone	human	R-970-5	CRL-7723*
Osteosarcoma	bone	human	TE 417.T	CRL-7765*
Osteosarcoma	bone	human	TE 418.T	CRL-7766*
Osteosarcoma	bone	human	TO 203.T	CRL-7780*
Osteosarcoma	bone	human	HT 728.T	CRL-7783*
Osteosarcoma	bone	human	Hs 14.T	CRL-7823*
Osteosarcoma	bone	human	T1-73	CRL-7943*
Osteosarcoma	bone	human	143B	CRL-8303†
Osteosarcoma	bone	human	143B PML BK TK	CRL-8304†
Osteosarcoma	bone	human	Saos-2	HTB-85
Osteosarcoma	bone	human	U-2 OS	HTB-96
Osteosarcoma	bone	rat	UMR-106	CRL-1661
Osteosarcoma	bone	rat	UMR-108	CRL-1663
Osteosarcoma	bone, connective tissue	dog	CF11.T	CRL-6217*
Osteosarcoma	bone, connective tissue	human	Hs 88.T	CRL-7060*
Osteosarcoma	bone, connective tissue	human	Hs 864.T	CRL-7600*
Osteosarcoma	thymus	cat	FC95.Thy	CRL-6114*
Osteosarcoma, multipotential sarcoma	bone	human	SJSA-1	CRL-2098
Papilloma	pharynx	human	Hs 840.T	CRL-7573*
Papilloma	skin	rabbit, cottontail	CTPS	CRL-6496*
Papilloma, squamous	skin	human	Hs 416.T	CRL-7289*
Papilloma, transitional cell	bladder, urinary	human	RT4	HTB-2
Pheochromocytoma	adrenal gland	rat	PC-12	CRL-1721
Pheochromocytoma	adrenal gland	rat	PC-12	CRL-1721
Plasmacytoma, myeloma	B lymphoblast	human	U266B1	TIB-196
Plasmacytoma, myeloma	B lymphocyte	mouse	MOPC-31C	CCL-130
Plasmacytoma, myeloma	B lymphocyte	mouse	MPC-11	CCL-167
Plasmacytoma, myeloma	B lymphocyte	mouse	P1.17	TIB-10
Plasmacytoma, myeloma	B lymphocyte	mouse	C1.18.4	TIB-11
Plasmacytoma, myeloma	B lymphocyte	mouse	HOPC 1F/12	TIB-13
Plasmacytoma, myeloma	B lymphocyte	mouse	MPC 11 OUA'	TIB-15
Plasmacytoma, myeloma	B lymphocyte	mouse	XC1.5/51	TIB-16
Plasmacytoma, myeloma	B lymphocyte	mouse	XS63	TIB-17
Plasmacytoma, myeloma	B lymphocyte	mouse	S194/5.XXO-1	TIB-19
Plasmacytoma, myeloma	B lymphocyte	mouse	MOPC 315	TIB-23
Plasmacytoma, myeloma	B lymphocyte	mouse	J558	TIB-6

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Tumor Cell Lines by Disease

Disease	Source	Species	Name	ATCC® No.
Plasmacytoma, myeloma	B lymphocyte	mouse	P3.6.2.8.1	TIB-8
Plasmacytoma, myeloma	B lymphocyte, peripheral blood	human	RPMI 8226	CCL-155
Plasmacytoma, myeloma	bone marrow, B lymphocyte	human	NCI-H929	CRL-9068†
Primitive neuroectodermal, malignant	retroperitoneal	human	SK-PN-DW	CRL-2139
Retinoblastoma	eye, retina	human	WERI-Rb-1	HTB-169
Retinoblastoma	eye, retina	human	Y79	HTB-18
Rhabdomyosarcoma	connective tissue	human	TE 441.T	CRL-7767*
Rhabdomyosarcoma	connective tissue	human	TE 617.T	CRL-7774*
Rhabdomyosarcoma	connective tissue	human	Hs 729.T	CRL-7862*
Rhabdomyosarcoma	mixed connective and soft	human	TE 381.T	CRL-7763*
Rhabdomyosarcoma	muscle	human	RD	CCL-136
Rhabdomyosarcoma	muscle	human	A-673	CRL-1598
Rhabdomyosarcoma	muscle	human	Hs 729	HTB-153
Rhabdomyosarcoma	muscle	human	A-204	HTB-82
Rhabdomyosarcoma	muscle, skeletal	human	Hs 94.T	CRL-7064*
Rhabdomyosarcoma	unknown	human	TE 159.T	CRL-7752*
Rhabdomyosarcoma	unknown	human	TE 125.T	CRL-7945*
Rhabdomyosarcoma, renal	kidney	human	Hs 926.T	CRL-7678*
Sarcoma	connective tissue	cat	FC100.T	CRL-6115*
Sarcoma	connective tissue	mouse	EHS	CRL-2108
Sarcoma	spleen	cat	FC100.Sp	CRL-6116*
Sarcoma	unknown	mouse	CCRF S-180 II	CCL-8
Sarcoma	unknown	mouse	Sarcoma 180	TIB-66
Sarcoma	unknown	rat	Jensen Sarcoma	CCL-45
Sarcoma	unknown	rat	RR1022	CCL-47
Sarcoma (anaplastic osteosarcoma or Ewing's sarcoma)	bone	human	SK-ES-1	HTB-86
Sarcoma or lymphoma	lung	human	Hs 57.T	CRL-7037*
Sarcoma, Ewing's	bone	human	Hs 822.T	CRL-7556*
Sarcoma, Ewing's	bone	human	Hs 863.T	CRL-7598*
Sarcoma, Ewing's	bone	human	RD-ES	HTB-166
Sarcoma, giant cell	bone	human	Hs 706.T	CRL-7447*
Sarcoma, giant cell	bone	human	Hs 737.T	CRL-7473*
Sarcoma, giant cell	bone	human	Hs 821.T	CRL-7554*
Sarcoma, giant cell	bone	human	Hs 846.T	CRL-7579*
Sarcoma, giant cell	bone	human	Hs 883.T	CRL-7617*
Sarcoma, giant cell	connective tissue	human	Hs 127.T	CRL-7081*
Sarcoma, giant cell	vertebral column	human	Hs 814.T	CRL-7547*
Sarcoma; heterozygous for tuberin; tuberous sclerosis model	unknown, cutaneous	mouse	tsc2 ang1	CRL-2620
Sarcoma, pagetoid	skin	human	Hs 925.T	CRL-7677*
Sarcoma, reticulum cell	bone marrow	cat	FC11.BM	CRL-6088*
Sarcoma, reticulum cell	B lymphocyte	mouse	A20	TIB-208
Sarcoma, reticulum cell	B lymphocyte	mouse	X16C8.5	TIB-209
Sarcoma, reticulum cell	lymph node	human	Hs 324.T	CRL-7239*
Sarcoma, reticulum cell	monocyte/macrophage	mouse	J774A.1	TIB-67
Sarcoma, spindle cell	connective tissue	human	Hs 132.T	CRL-7085*
Sarcoma, synovial	connective tissue	human	Hs 701.T	CRL-7440*
Sarcoma, synovial	synovium	human	SW 982	HTB-93
Sarcoma, uterine	uterus	human	MES-SA	CRL-1976
Sarcoma, uterine	uterus	human	MES-SA/Dx5	CRL-1977
Sarcoma, uterine	uterus	human	MES-SA/MX2	CRL-2274
Schwannoma	Schwann cell	rat	RT4-D6P2T	CRL-2768
Teratocarcinoma	stem cell, nullipotent	human	NCCIT	CRL-2073
Teratocarcinoma	testis	mouse	NULLI-SCC1	CRL-1566
Teratocarcinoma, carcinoma	embryo	mouse	P19	CRL-1825

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Tumor Cell Lines by Disease

Disease	Source	Species	Name	ATCC® No.
Teratocarcinoma, carcinoma	embryonal stem cell, nullipotent	mouse	NE	CRL-2070
Teratocarcinoma, carcinoma	embryonal stem cell, nullipotent	mouse	NF-1	CRL-2075
Teratocarcinoma, carcinoma	embryonal stem cell, pluripotent	mouse	SCC-PSA1	CRL-1535
Teratoma	keratinocyte	mouse	XB-2	CL-177
Teratoma	ovary	human	TE 84.T	CRL-7944*
Teratoma, sacrococcygeal	bone	human	TE 76.T	CRL-7732*
Teratoma, sacrococcygeal	bone	human	TE 130.T	CRL-7746*
Tumor	bladder, urinary	rat	NBT-II	CRL-1655
Tumor	lung, transformed	mouse	TC-1	JHU-1
Tumor	mammary gland	mouse	4T1	CRL-2539
Tumor	mammary gland	mouse	MMT 060562	CCL-51
Tumor	mammary gland	mouse	C127I	CRL-1616
Tumor	mammary gland	mouse	C127:LT	CRL-1804
Tumor	pancreas, exocrine	rat	AR42J	CRL-1492
Tumor	pancreas, exocrine	rat	ARIP	CRL-1674
Tumor	pituitary	mouse	AtT-20	CCL-89
Tumor	pituitary	mouse	AtT-20/D16v-F2	CRL-1795
Tumor	pituitary	rat	GH_1	CCL-82
Tumor	pituitary	rat	GH_3	CCL-82.1
Tumor	pituitary	rat	GH_4C_1	CCL-82.2
Tumor	pituitary	rat	MMQ	CRL-10609†
Tumor	spleen	mouse	MM7-11.Sp	CRL-6381*
Tumor	unknown	dog	A-72	CRL-1542
Tumor, AMLV induced	monocyte	mouse	WEHI-274.1	CRL-1679
Tumor, AMLV induced	monocyte	mouse	WEHI-265.1	TIB-204
Tumor, AMLV induced	monocyte/macrophage	mouse	RAW 309 Cr.1	TIB-69
Tumor, AMLV induced	monocyte/macrophage	mouse	WR19M.1	TIB-70
Tumor, AMLV induced	monocyte/macrophage	mouse	RAW 264.7	TIB-71
Tumor, DMBA induced	unknown	rat	Rn6T	CRL-6601*
Tumor, acinar cell	pancreas	mouse	266-6	CRL-2151
Tumor, chemically induced	unknown	rat	Rn 3T	CRL-6511*
Tumor, chemically induced	unknown	rat	Rn 4T	CRL-6512*
Tumor, glucose-stimulated insulin release	pituitary	mouse	AtT-20ins (CGT-6)	CRL-11285†
Tumor, islet cell, insulinoma	pancreas	mouse, transgenic	TGP61	CRL-2135
Tumor, islet cell, insulinoma	pancreas	mouse, transgenic	TGP52	CRL-2140
Tumor, Leydig cell	testis	mouse	I-10	CCL-83
Tumor, Leydig cell	testis	mouse	MLTC-1	CRL-2065
Tumor, Leydig cell	testis	rat	LC-540	CCL-43
Tumor, Leydig cell	testis	rat	R2C	CCL-97
Tumor, malignant primitive neuroectodermal	brain, cerebellum	human	PFSK-1	CRL-2060
Tumor, nonneoplastic	oral	human	Hs 53.T	CRL-7033*
Tumor, premalignant	mammary gland	mouse	CL-S1	CRL-1615
Tumor, rhabdoid	kidney	human	G-401	CRL-1441
Tumor, small cell	pancreas	mouse, transgenic	TGP55	CRL-2150
Tumor, smooth muscle-like, methylnitrosourea induced	brain	mouse	BC_3H1	CRL-1443
Xanthogranuloma	skin	human	Hs 156.T	CRL-7102*

* Part of the NBL collection; see page 12. † Patent item; see page 12.
See the ATCC online catalog for the complete description of a cell line.

Glycobiology vol. 12 no. 8 pp. 485–497, 2002

Preferential binding of the anticancer drug rViscumin (recombinant mistletoe lectin) to terminally α2-6-sialylated neolacto-series gangliosides

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Received on February 25, 2002; revised on April 12, 2002; accepted on April 17, 2002

Production of biochemically defined recombinant mistletoe lectin was achieved by cloning and separate expression of the single catalytically active A-chain and the B-chain with carbohydrate binding properties in *Escherichia coli*, yielding an active heterodimeric protein named rViscumin (Eck *et al.* [1999] *Eur. J. Biochem.*, 265, 788–797). Employing solid phase binding assays, rViscumin was shown to preferentially bind to terminally α2-6-sialylated neolacto-series gangliosides IV^{6}Neu5Ac-nLc4Cer, VI^{6}Neu5Ac-nLc6Cer, and VIII^{6}Neu5Ac-nLc8Cer isolated from human granulocytes. Only marginal binding of rViscumin to galactose-terminated neutral GSLs was determined, whereas reinvestigation of ricin specificity demonstrated this lectin as a galactose-binding protein. Human promyelotic HL-60 cells exhibited an IC_{50} value (half maximum cytotoxicity) of 1.16 pM and human bladder carcinoma 5637 cells of 12.1 pM rViscumin; CHO-K1 cells were resistant to rViscumin treatment up to a concentration of 5.26 nM tested. Quantification of the predominant receptor ganglioside IV^{6}Neu5Ac-nLc4Cer by means of a specific anti-Neu5Acα2-6Galβ1-4GlcNAc-R antibody revealed 3.68×10^6 and 1.54×10^6 receptor molecules per HL-60 and 5637 cell, respectively; CHO-K1 cells were negative, lacking α2-6-sialylated gangliosides. The data imply a direct correlation of rViscumin cytotoxicity and the expression of receptor ganglioside. Moreover, CHO-K1 cells were rendered susceptible toward rViscumin cytotoxicity after exogenous application of human granulocyte gangliosides. Thus, (1) rViscumin has to be considered as a sialic acid–specific rather than a galactose-specific type II ribosome-inactivating protein, and (2) neolacto-series gangliosides with Neu5Acα2-6Galβ1-4GlcNAc-terminus are true functional and physiologically relevant rViscumin receptors.

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Key words: gangliosides/glycosphingolipids/recombinant viscumin/ricin/TLC immunostaining

Introduction

Proprietary mistletoe extracts are widely used as therapeutic immunomodulatory agents, mainly for oncological application in adjuvant therapy but also for general immunostimulation (Bocci, 1993; Hajto *et al.*, 1990; Beuth, 1997). Mistletoe lectin (ML) is a ribosome-inactivating protein of type II like ricin, abrin, and others (Barbieri *et al.*, 1993). Although these toxic proteins are of different phylogenetic origin, they share similar activities and structural properties (Eschenburg *et al.*, 1998), being potent inhibitors of eukaryotic protein synthesis at the ribosomal level (Glück *et al.*, 1992).

Three MLs with different carbohydrate specificities (ML-I, ML-II, and ML-III) have been isolated from mistletoe leaves. ML-I (also known as viscumin) was identified as the main therapeutic principle (Hajto *et al.*, 1989; Beuth *et al.*, 1993). Unlike ricin, ML-I-containing extracts are medically applied for the treatment of human cancer. Viscumin is categorized as heterodimeric two-chain (type II) ribosome-inactivating protein, composed of a catalytically active A chain with RNA N-glycosidase activity and a B-chain with carbohydrate binding specificities (Olsnes *et al.*, 1982; Franz, 1986) to yet undefined receptors on the surface of target cells. Recently, biotechnological production of recombinant ML (rViscumin) was achieved after cloning and separate expression of the single chains in *Escherichia coli* (Eck *et al.*, 1999a,b), yielding the active rML-heterodimer (Eck *et al.*, 1999b). The enzymatic A-chain activity and the carbohydrate binding activity of the B-chain are both essential for the cytotoxic apoptosis-inducing effect of the holoprotein as an anticancer drug (Langer *et al.*, 1999).

In numerous studies ML has been reported to belong to the group of galactoside-specific/lactose-binding lectins, which bind and cross-link certain multiantennary oligosaccharides, glycopeptides, and glycoproteins (Hajto *et al.*, 1989; Gabius *et al.*, 1992; Barbieri *et al.*, 1993; Gupta *et al.*, 1996). Due to the lack of data concerning the potency of glycosphingolipids (GSLs) as lectin receptors, we investigated the binding specificity of rViscumin in comparison to ricin toward neutral GSLs and gangliosides.

GSLs are amphipathic molecules consisting of a hydrophilic oligosaccharide chain and a hydrophobic component named ceramide (Stults *et al.*, 1989). Gangliosides are characterized by the presence of one or more sialic acids in the oligosaccharide moiety, which are known to play crucial roles in various biological functions (Varki, 1992; Schauer *et al.*, 1995).

Gangliosides are involved in cell–cell recognition (Hakomori et al., 1998; Hakomori, 2002) and adhesion by counterpart lectins (Schnaar, 1991; Feizi, 2001) and are well-characterized cellular attachment sites for viruses, bacteria, and exotoxins (Suzuki, 1994; Karlsson, 1989). Employing naturally occurring GSLs of mammalian origin, we were able to show herein the preferential binding of rViscumin to α2-6-sialylated neolacto-series gangliosides and only marginal binding to galactose-terminated neutral GSLs. Consequently, rViscumin has to be considered as a sialic acid–specific rather than as a galactose-specific type II ribosome-inactivating protein, whereas reinvestigation of ricin specificity confirmed this lectin as galactose-specific.

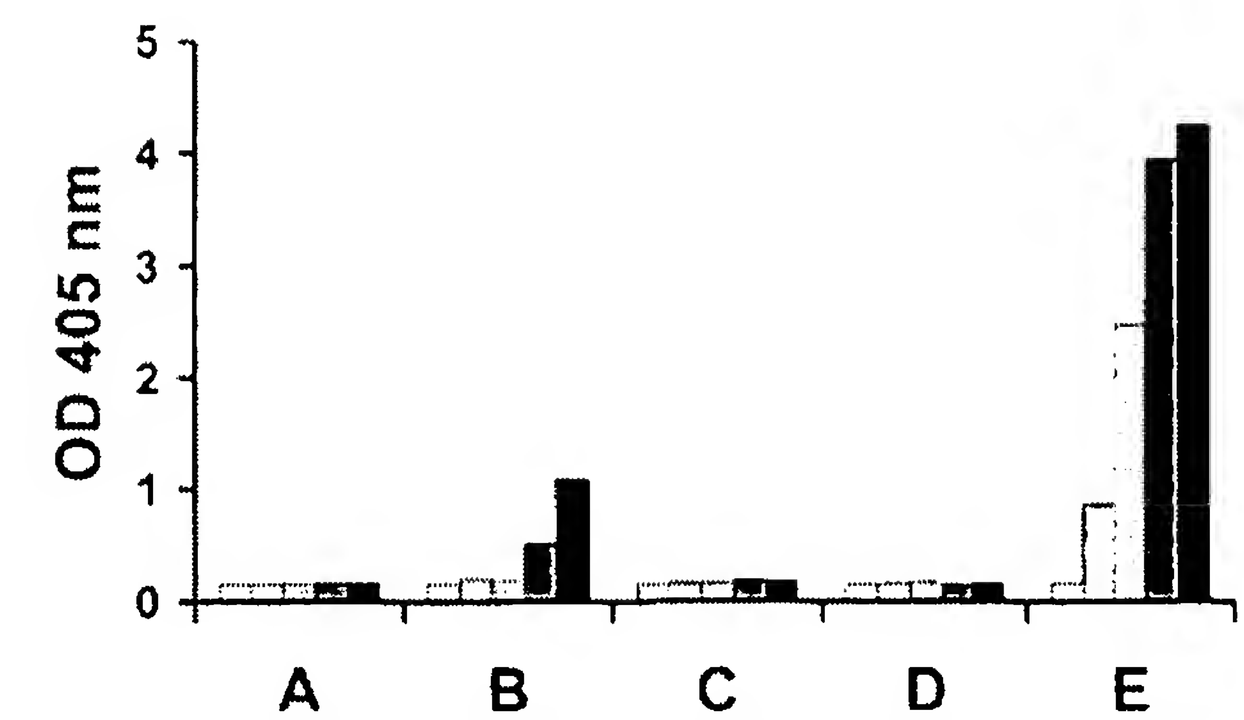

Fig. 1. Microwell adsorption assay of rViscumin with neutral GSLs and gangliosides. Amounts applied correspond to bars from left to right for each GSL fraction. (**A**) Neutral GSLs from human erythrocytes: 0, 1.25, 2.5, 5, and 10 μg; (**B**) neutral GSLs from human granulocytes: 0, 1.88, 3.75, 7.5, and 15 μg; (**C**) neutral GSLs from MDAY-D2 cells: 0, 2.5, 5, 10, and 20 μg; (**D**) human brain gangliosides: 0, 1.25, 2.5, 5, and 10 μg; (**E**) HGGs: 0, 1.25, 2.5, 5, and 10 μg. The orcinol-stained thin-layer chromatogram of neutral GSLs (**A**, **B**, and **C**) is shown in Figure 8; the resorcinol-stained TLC runs of both human brain gangliosides (**D**) and HGG (**E**), are displayed in Figures 3A and 6A. For structures see Tables I and II.

Results

The first approach to obtain preliminary structural information regarding GSL binding specificities of rViscumin was to perform a screening by use of microwells coated with GSL mixtures of well-defined structures. Human erythrocytes are known to express globo-series neutral GSLs Gb4Cer and Gb3Cer, the former being the dominant one. Neutral GSLs of human granulocytes preferentially contain LacCer and the neolacto-series GSLs nLc4Cer, nLc6Cer, and to a lesser extent fucosylated derivatives of, for example, nLc6Cer, the so-called Lewis^x-GSLs. By searching for certain ganglio-series neutral GSLs as potential receptors, a GSL mixture of MDAY-D2 cells was chosen due to its high content of Gg3Cer and Gg4Cer. Human brain gangliosides are suitable to investigate binding specificities of ganglio-series gangliosides, and human granulocyte gangliosides (HGGs) are the ideal candidates exploring the binding potency of terminally α2-3- and α2-6-sialylated neolacto-series monosialogangliosides.

Binding of rViscumin toward microwell adsorbed neutral GSLs and gangliosides

The microwell adsorption assays of rViscumin with three reference neutral GSL preparations (A, B, and C) and two ganglioside mixtures of well-known structures (D and E) are shown in Figure 1. The only positive reaction within the neutral GSL fractions could be detected for neutral GSLs from human granulocytes, consisting of LacCer and nLc4Cer as the main compounds (Figure 1B). Of the two ganglioside fractions, human brain gangliosides were negative, but HGG revealed strong binding interaction with rViscumin (Figure 1E).

TLC overlay assay of rViscumin with granulocyte neutral GSLs and gangliosides

To identify the individual GSLs responsible for rViscumin binding, thin-layer chromatography (TLC) overlay assays with the faint positive neutral GSLs from human granulocytes and the strongly positive HGG were performed. The TLC-binding assay of rViscumin with neutral GSLs of human granulocytes revealed weak reaction with LacCer (Figure 2A, lane b), which represents the dominant GSL in this mixture. While LacCer (Galβ1-4Glcβ1-1Cer) was found to be at least a low-affinity binding ligand for rViscumin (see Table I), the N-acetyl group of the penultimate GlcNAc completely abolished rViscumin binding toward nLc4Cer and nLc6Cer (both carrying the Galβ1-4GlcNAc-disaccharide at the nonreducing terminus).

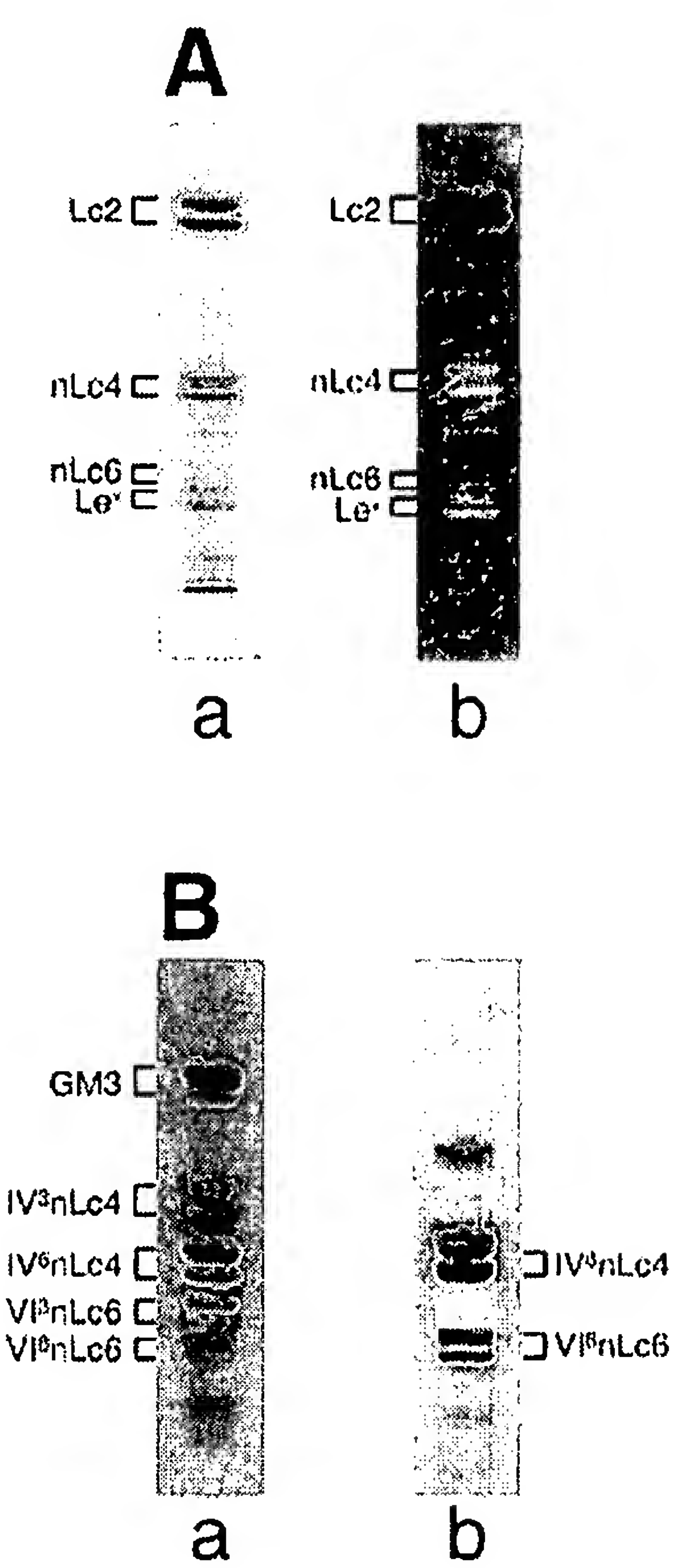

Fig. 2. TLC overlay binding assay of rViscumin with neutral GSLs (**A**) and gangliosides (**B**) from human granulocytes. (**A**) Lane a: orcinol-stained chromatogram of 15 μg neutral GSLs; lane b: corresponding rViscumin overlay assay. (**B**) Lane a: resorcinol-stained chromatogram of 15 μg HGGs; lane b: corresponding rViscumin overlay assay.

Table I. Binding of rViscumin and ricin toward neutral GSLs

Structure	Abbreviation	rViscumin*	Ricin*
Galβ1-4Glcβ1-1Cer	Lc2	+	+
Galα1-4Galβ1-4Glcβ1-1Cer	Gb3	–	(+)
GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer	Gb4	–	–
GalNAcβ1-4Galβ1-4Glcβ1-1Cer	Gg3	–	–
Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer	Gg4	–	+++
Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer	nLc4	–	+++++
Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer	nLc6	–	+++++
Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer	Lewis^x	–	–

For TLC immunostain of rViscumin see Figure 2A and of ricin see Figure 8.
*Appearance on immunostained TLCs graded from – (negative), (+) trace positivity, + weak binding up to +++++ highest intensity.

Fucosylated neolacto-series GSLs (Lewis^x-GSLs) were negative, too. It should be mentioned that this faint binding of LacCer could only be obtained by "overstaining" the TLC plate (20 h overnight dye incubation), indicated by the intensive background staining (Figure 2A, lane b).

On the other hand, a fast and clear reaction could be achieved with HGG, uncovering two strongly stained double bands (Figure 2B, lane b). Compared with the resorcinol stain of the respective lipids, the terminally α2-6-sialylated neolacto-series gangliosides IV^6nLc4Cer and VI^6nLc6Cer were suggested to represent the preferential receptors of rViscumin.

TLC overlay assays of anti-IV^6nLc4Cer antibody and rViscumin with HPLC-separated neolacto-series gangliosides

To confirm this hypothesis, rViscumin binding assays were performed with high-performance liquid chromatography (HPLC)-separated HGG-fractions. Ganglioside fractions (Figure 3A) comprising IV^3nLc4Cer (HGG1, lane c), VI^3nLc6Cer (HGG2, lane d), IV^6nLc4Cer (HGG3, lane e), and IV^6nLc4Cer plus VI^6nLc6Cer and $VIII^6$nLc8Cer (HGG4, lane f) were chromatographed and overlayed with an anti-Neu5Acα2-6Galβ1-4GlcNAc-R antibody, which has been raised by immunizing a chicken with IV^6nLc4Cer. As shown in Figure 3B, the antibody specifically bound to IV^6nLc4Cer, VI^6nLc6Cer, and $VIII^6$nLc8Cer of the HPLC fractions as well as to these gangliosides in the total HGG fraction. The isomeric structures IV^3nLc4Cer and VI^3nLc6Cer were not detected by this antibody. A further indication for the antibody specificity is shown in lane a of Figure 3B, where no cross-reaction could be observed with any ganglio-series gangliosides. Exactly identical binding patterns compared to the anti-Neu5Acα2-6Galβ1-4GlcNAc-R antibody were obtained in the TLC overlay binding assay with rViscumin (Figure 3C), exhibiting its high specificity toward monosialogangliosides with Neu5Acα2-6Galβ1-4GlcNAc-termini (see Table II). Due to assaying ganglioside fractions isolated by gradient anion-exchange HPLC, any cross-reactivities to, for example, "underlying" disialogangliosides or other lipid contaminants, which are absent in the HPLC eluates but might occur in the total HGG fraction, could be excluded. Thus, the most important and unexpected result of these binding assays is the fact that

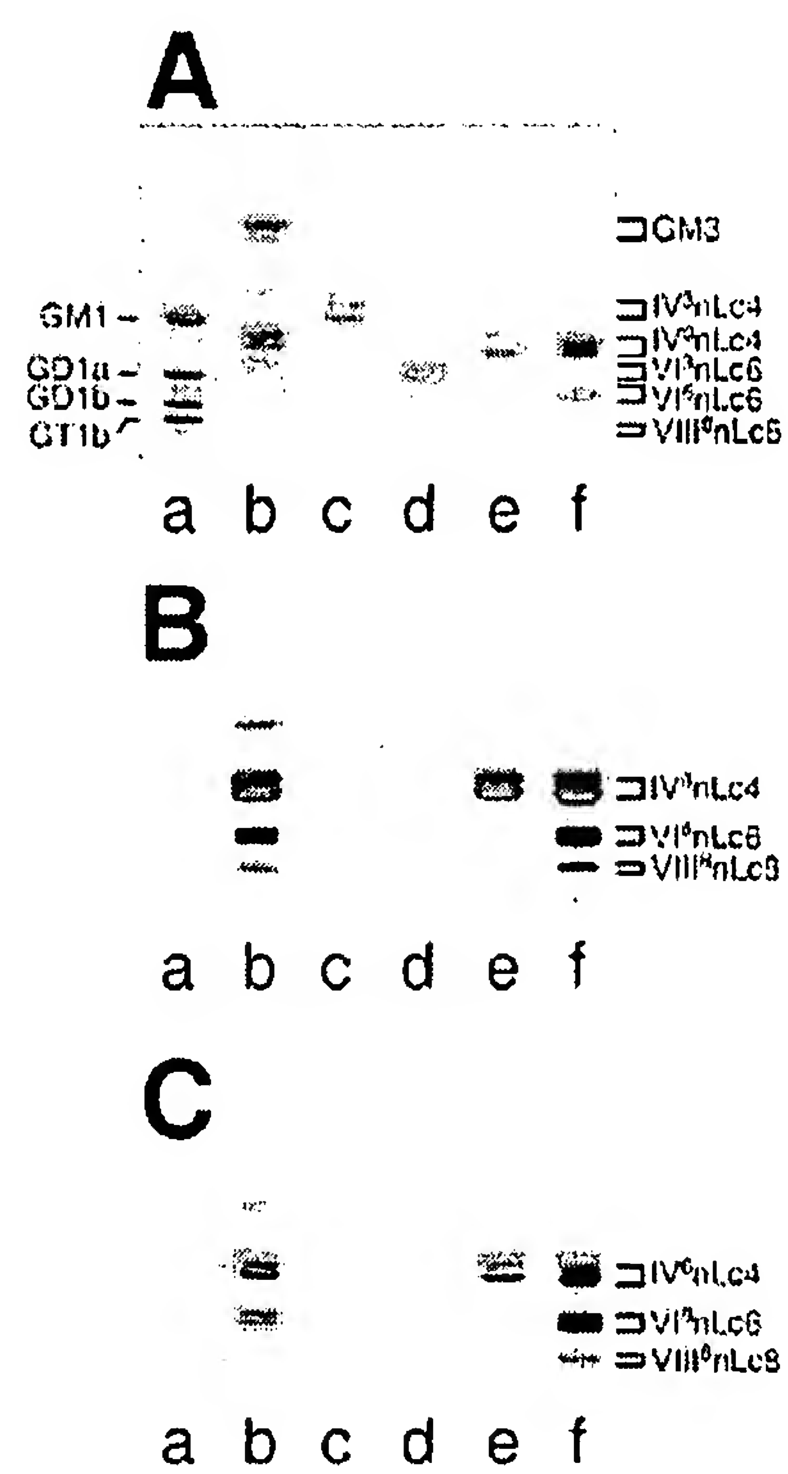

Fig. 3. Resorcinol stain (**A**), anti-IV^6nLc4Cer antibody TLC overlay assay (**B**), and rViscumin TLC overlay assay (**C**) with HPLC-purified α2-3- and α2-6-sialylated neolacto-series monosialogangliosides. Lanes a: 15 μg human brain gangliosides; lanes b: 15 μg human HGGs; lanes c: 4 μg IV^3nLc4Cer (HGG1); lanes d: 4 μg VI^3nLc6Cer (HGG2); lanes e: 4 μg IV^6nLc4Cer (HGG3); lanes f: 8 μg IV^6nLc4Cer, VI^6nLc6Cer, and $VIII^6$nLc8Cer (HGG4).

rViscumin is not a galactose-specific but a sialic acid–specific lectin with strict preference of the Neu5Acα2-6Galβ1-4GlcNAc configuration.

NanoESI-QTOF MS/MS of α2-6-sialylated gangliosides

The molecular ions of terminally α2-6-sialylated neolacto-series gangliosides obtained from the mass spectrometry (MS) spectrum of fraction HGG4 are listed in Table III. The most

Table II. Binding of rViscumin and ricin toward gangliosides

Structure	Abbreviation	rViscumin*	Ricin*
Neu5Acα2-3Galβ1-4Glcβ1-1Cer	GM3	–	–
Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glcβ1-1Cer	GM1	–	(+)
Neu5Acα2-3Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glcβ1-1Cer	GD1a	–	–
Galβ1-3GalNAcβ1-4(Neu5Acα2-8Neu5Acα2-3)Galβ1-4Glcβ-1Cer	GD1b	–	–
Neu5Acα2-3Galβ1-3GalNAcβ1-4(Neu5Acα2-8Neu5Acα2-3)Galβ1-4Glcβ1-1Cer	GT1b	–	–
Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer	IV³nLc4	–	–
Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer	IV⁶nLc4	+++++	–
Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer	VI³nLc6	–	–
Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer	VI⁶nLc6	+++++	–

For TLC immunostain of rViscumin see Figures 2B and 3C and of ricin see Figure 9.
*Appearance of immunostained TLCs graded from – (negative), (+) trace positivity, + weak binding up to +++++ highest intensity.

Table III. Molecular ions of terminally α2-6-sialylated neolacto-series gangliosides of fraction HGG4 investigated in negative ion mode

Symbol	Structure	$[M-H]^-$
IV⁶nLc4	IV⁶Neu5Ac-nLc4Cer (d18:1/16:0)	1516.83
IV⁶nLc4	IV⁶Neu5Ac-nLc4Cer (d18:1/24:1)	1626.93
VI⁶nLc6	VI⁶Neu5Ac-nLc6Cer (d18:1/16:0)	1881.95
VI⁶nLc6	VI⁶Neu5Ac-nLc6Cer (d18:1/24:1)	1992.06
VIII⁶nLc8	VIII⁶Neu5Ac-nLc8Cer (d18:1/16:0)	2247.10
VIII⁶nLc8	VIII⁶Neu5Ac-nLc8Cer (d18:1/24:1)	2357.10

prominent ions (*m/z* 1516.83 and *m/z* 1626.93) belong to the monosialylated neolactotetraosylceramide species (Müthing *et al.*, 1993) as expected from the orcinol stain (Figure 3A, lane f) and the TLC immunodetection with the anti-Neu5Acα2-6Galβ1-4GlcNAc-antibody (Figure 3B, lane f). The signal intensities of those with increasing molecular size (that is, with nLc6Cer- and nLc8Cer-cores) and those that have not been characterized in detail before were lower. All ganglioside components were characterized by ceramide parts containing in general two major fatty acid moieties for one core carbohydrate structure contributing to the complexity of the spectrum (not shown).

For structural identification of nLc6Cer- and nLc8Cer-species, two molecular ions (*m/z* 1881.95 and *m/z* 2247.10, see Table III) were selected for low-energy collision-induced dissociation experiments. Their masses, together with the TLC immunostaining data were consistent with their assignment as being the gangliosides VI⁶Neu5Ac-nLc6Cer- and VIII⁶Neu5Ac-nLc8Cer-species, respectively, containing $C_{16:0}$ fatty acid in the ceramide portion.

A series of high abundance Y-type ions is present in the tandem mass spectrometry (MS/MS) spectrum of the molecular ion (*m/z* 1881.95) of the heptasaccharide ceramide (Figure 4A), starting with loss of sialic acid (Y_6 at *m/z* 1590.83) and followed by consecutive glycosidic cleavage of the other monosaccharide residues (Figure 4B). The mass differences of 162 Da between Y_6 (*m/z* 1590.83) and Y_5 (*m/z* 1428.80), and 203 Da between Y_5 (*m/z* 1428.80) and Y_4 (*m/z* 1225.69), are typical of the loss of either a hexose (e.g., galactose) or N-acetylhexosamine (e.g., N-acetylglucosamine), respectively. The sequence is completed by the ceramide ion Y_0 at *m/z* 536.50. Confirmatory evidence for the nLc6 core–containing terminal sialic acid is provided by the full series of B-type ions from the nonreducing end of the sugar chains. The B_1 ion (*m/z* 290.11) represents a single N-acetylneuraminic acid moiety, and B_2 (*m/z* 452.14) is assigned to the disaccharide sequence Neu5Ac-Hex. In this way the information obtained from Y-type ion series is supported by the B series ions up to the B_7 ion (*m/z* 1344.45) that represents the complete oligosaccharide core without ceramide. Additionally, a number of internal double cleavages give evidence for the sequence of oligosaccharide units in the chain like the Y_6/B_3 ion at *m/z* 364.12 (HexNAcHex), the Y_6/B_5 ion at *m/z* 729.25 ($Hex_2HexNAc_2$) or the low abundant Y_6/B_6 ion at *m/z* 891.32 ($Hex_3HexNAc_2$).

The fragmentation pattern in the spectrum of the molecular ion (*m/z* 2247.10) of the nonasaccharide ceramide is very similar to that of the heptasaccharide. A prominent Y-type ion series and a supporting but weaker B-type ion series provide corroborative evidence of an nLc8 core structure carrying a sialic acid moiety on its nonreducing terminus.

Biological activity of rViscumin

To investigate the cytotoxic ability of rViscumin on the cellular level, three cell lines with different biological background were employed to determine their cell-specific rViscumin sensitivity. Cultures of the human promyelocytic HL-60 cell line, consisting mainly of neutrophilic promyelocytes, exhibited the lowest IC_{50} value (highest sensitivity) responding to 1.16 pM rViscumin with half maximum cytotoxicity (Figure 5). The human 5637 cell line, representing small malignant cells with epithelial-like morphology and derived from bladder carcinoma, was one order of magnitude less susceptible corresponding to an IC_{50} value of 12.1 pM rViscumin. Chinese hamster ovary (CHO-K1) cells, epithelial cells derived from a hamster ovary, were insensitive toward the cytotoxic action of rViscumin up to the highest concentration of 5.26 nM tested in this series of experiments. At concentrations of 0.175 nM rViscumin, both HL-60 and 5637 cells completely lost viability, whereas CHO-K1

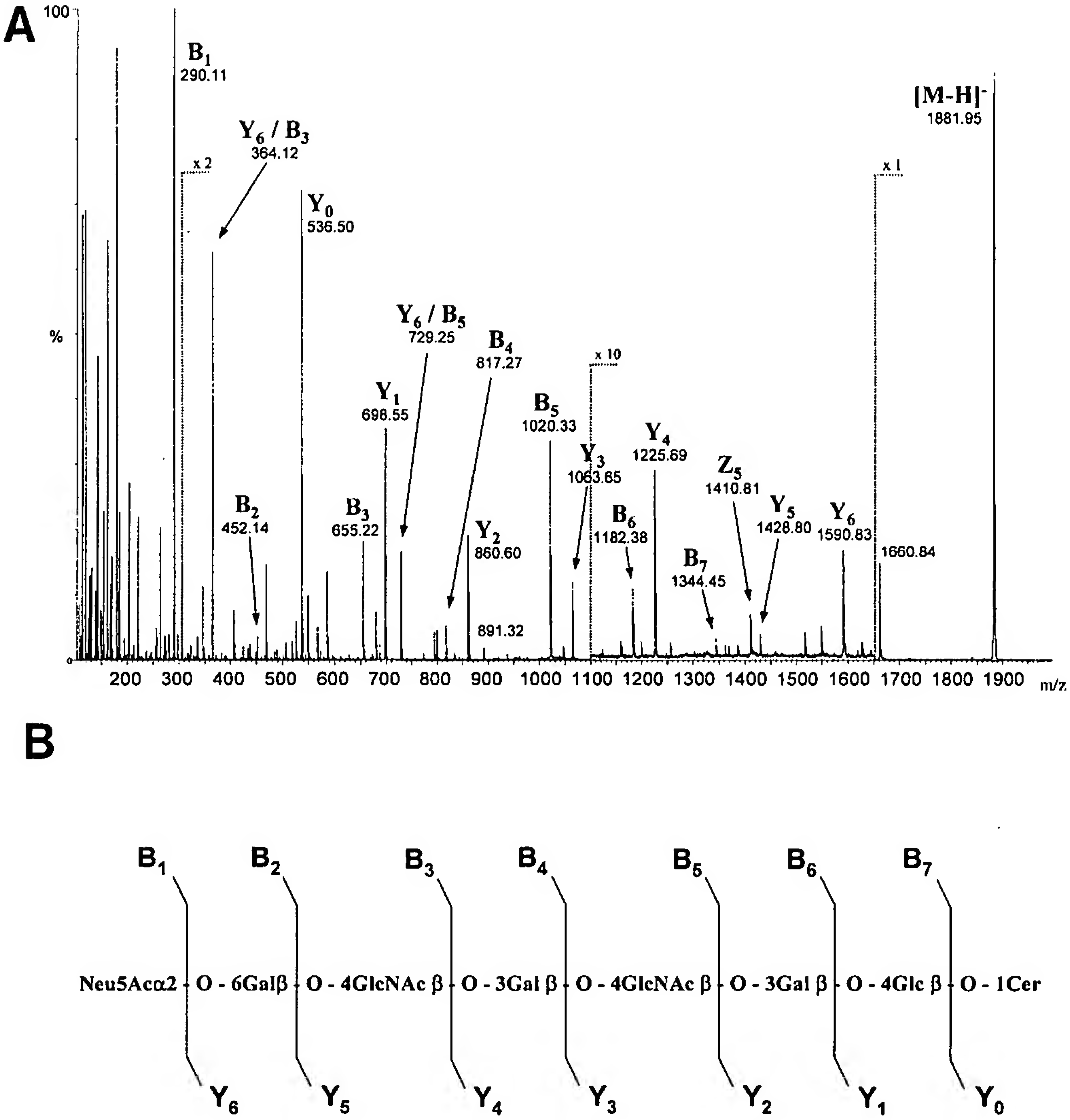

Fig. 4. Negative ion mode MS/MS spectrum (A) and fragmentation scheme with the proposed structure (B) of the ganglioside VI^{6}Neu5Ac-nLc6Cer (d18:1/16:0) with the molecular mass *m/z* 1881.95 from HGG fraction HGG4. The resorcinol-stained TLC run and TLC immunostains of HGG4 are displayed in Figure 3.

cells did not show any reduction in cell viability even at 30-fold higher concentrations.

Expression of rViscumin receptor ganglioside IV^{6}nLc4Cer by in vitro propagated CHO-K1, 5637, and HL-60 cells

Gangliosides were isolated from the three cell lines with different sensitivity and their orcinol-stained thin-layer chromatograms are shown in Figure 6A. A simple double-banded GM3 pattern is characteristic for CHO-K1 cells (Figure 6A, lane b), lacking any higher gangliosides. In contrast to CHO-K1 cells, 5637 cells exhibited a couple of complex and yet unknown gangliosides (Figure 6A, lane c). HL-60 cells were found to express rather low levels of gangliosides (Figure 6A, lane d) but resembling the ganglioside profile of human granulocytes (Figure 6A, lane a). The presence of IV^{6}nLc4Cer in the ganglioside fractions of the three cell lines was investigated by use of the anti-IV^{6}nLc4Cer antibody. For direct comparison, ganglioside amounts equivalent to 1×10^7 cells of each cell type were used for the TLC immunostain (Figure 6B). The rViscumin-resistent CHO-K1 cells were found to lack the receptor ganglioside, whereas 5637 and HL-60 cells express IV^{6}nLc4Cer structures, which appear as double bands on the immunostained chromatogram. Owing to their identical TLC positions compared to IV^{6}nLc4Cer of human granulocytes (Figure 6B, lane a), upper bands of both cell lines represent the typical granulocyte gangliosides IV^{6}nLc4Cer with C24-fatty acid (compound 1) and lower bands IV^{6}nLc4Cer with C16-fatty acid (compound 2). Applying 134 ng of total HGG, the absolute amounts of compound 1 (26% of total HGG) and compound 2 (13.3% of total HGG) of lane a in Figure 6B correspond to 34.8 ng and 17.8 ng, respectively. Deduced from the TLC overlay assay, CHO-K1 cells were receptor-negative and HL-60 cells exhibited considerably higher quantities of IV^{6}nLc4Cer compared with 5637 cells. These findings are in excellent

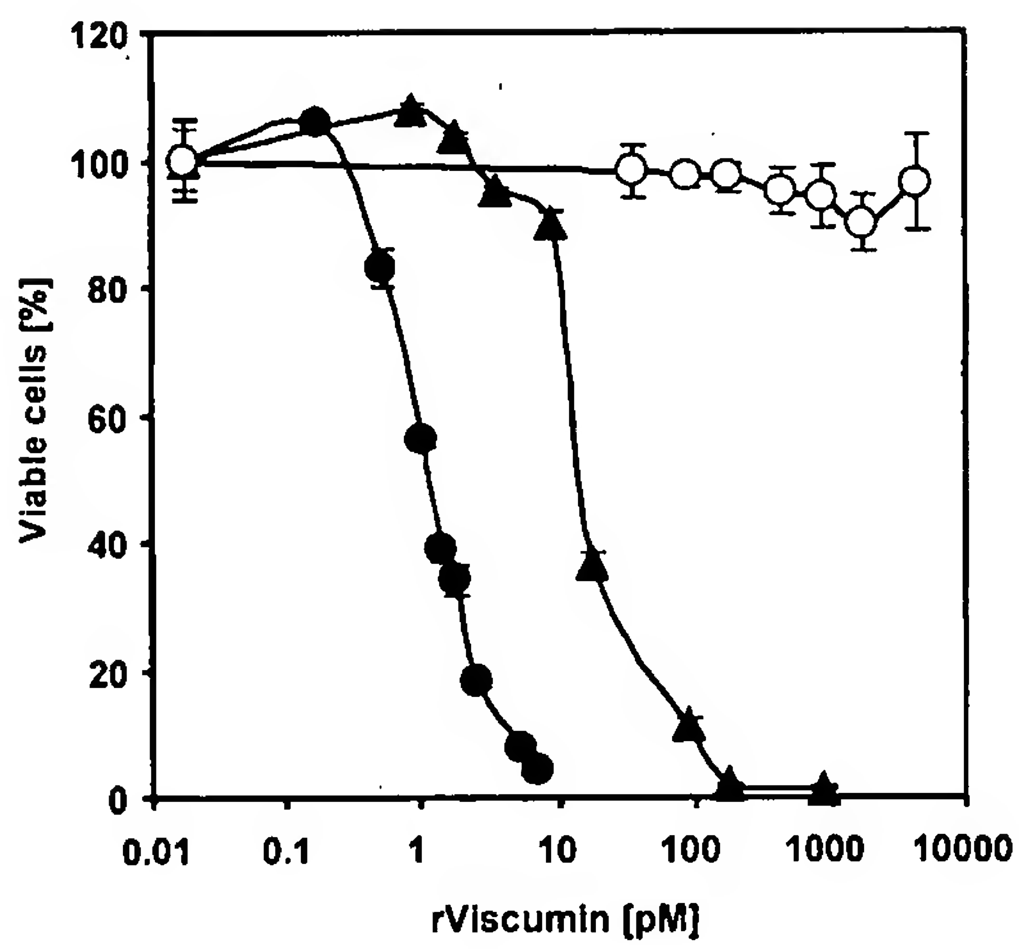

Fig. 5. Determination of the biological activity of rViscumin. Viability of HL-60 cells (closed circles), 5637 cells (triangles), and CHO-K1 cells (open circles) was plotted versus rViscumin concentration. IC_{50} values were determined to be 1.16 pM for HL-60 and 12.1 pM for 5637 cells. CHO-K1 cells were insensitive up to a concentration of 5.26 nM tested. Viability was measured in the WST-1 cytotoxicity assay and calculated as percentage relative to control cell cultures without rViscumin treatment.

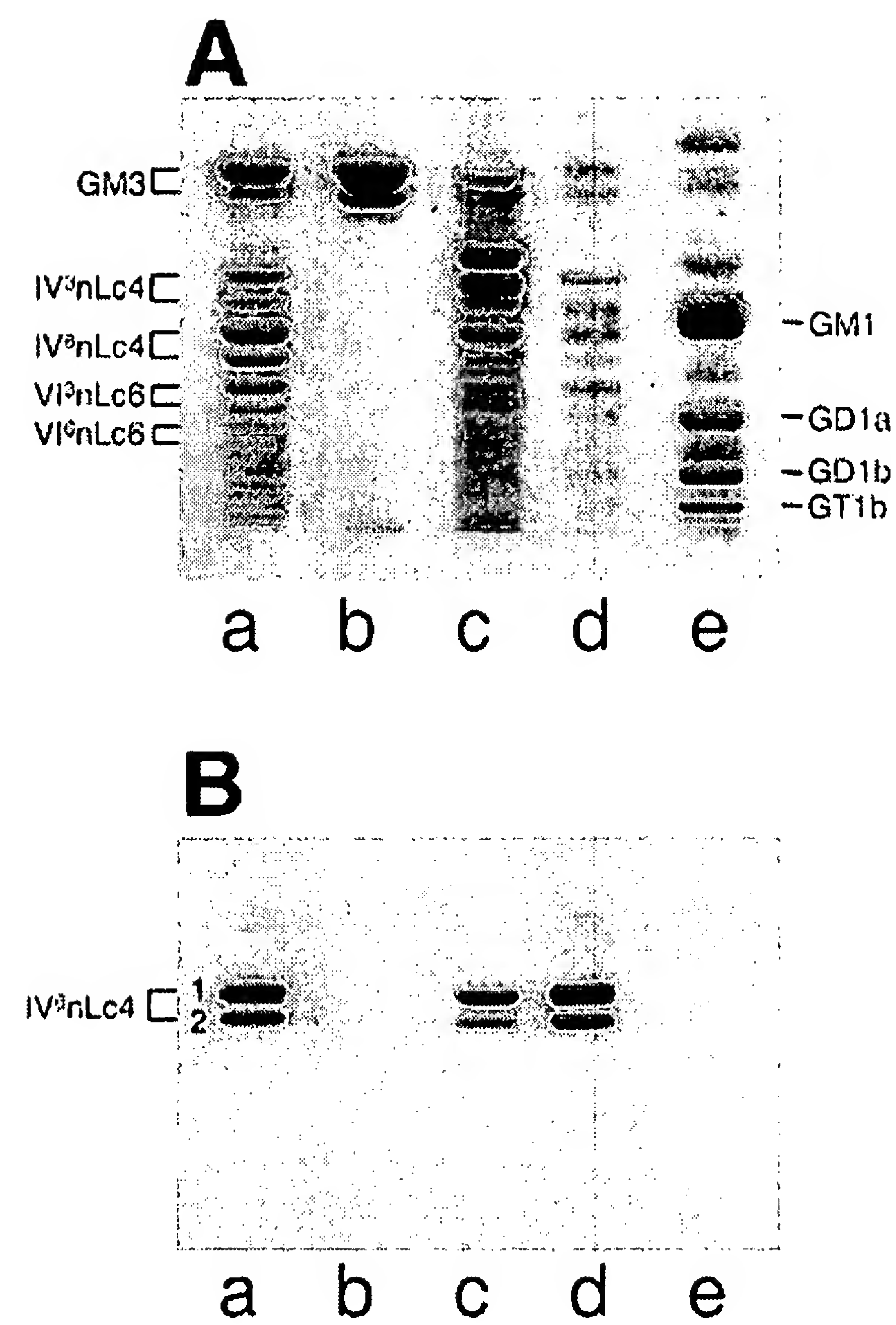

Fig. 6. Orcinol stain (**A**) and anti-IV^{6}nLc4Cer antibody TLC overlay assay (**B**) with *in vitro* propagated cell lines. (**A**) Lane a: 7 µg HGGs; ganglioside amounts corresponding to 1 × 10^7 CHO-K1 cells, 4 × 10^7 5637 cells, and 4 × 10^7 HL-60 cells were applied on lanes b, c, and d, respectively; lane e: 10 µg human brain gangliosides. (**B**) Lane a: 0.134 µg HGGs; ganglioside amounts equivalent to 1 × 10^7 CHO-K1, 5637, and HL-60 cells were applied on lanes b, c, and d, respectively; lane e: 10 µg human brain gangliosides. Compound 1 (lane a): IV^{6}nLc4Cer (C24-fatty acid); compound 2 (lane a): IV^{6}nLc4Cer (C16-fatty acid). For structures of gangliosides see Table II and for quantification of IV^{6}nLc4Cer by TLC immunostaining with anti-IV^{6}nLc4Cer antibody see Table IV.

agreement with rViscumin resistance of CHO-K1 cells and the significantly higher rViscumin sensitivity of HL-60 cells in comparison to 5637 cells (see Figure 5), pointing to an obvious correlation of cellular sensitivity and expression of receptor gangliosides.

Quantification of receptor ganglioside IV^{6}nLc4Cer in 5637 and HL-60 cells

To calculate the absolute amounts of IV^{6}nLc4Cer receptor gangliosides per single cells, TLC immunostained bands of defined cell numbers, ranging from 1 × 10^6 to 2 × 10^7 cells, were quantified by densitometry with the aid of HGG calibration curves. Based on the molecular weights of IV^{6}nLc4Cer with C24-fatty acid (compound 1, MW = 1626) and IV^{6}nLc4Cer with C16-fatty acid (compound 2, MW = 1516) and Avogadro's constant, the absolute molecule numbers per single cell were determined for 5637 and HL-60 cells (see Table IV). For 5637 and HL-60 cells 1.54 × 10^6 and 3.68 × 10^6 IV^{6}nLc4Cer (compound 1 plus compound 2) receptor molecules per cell were revealed, respectively. As a final result, the absolute amount of IV^{6}nLc4Cer was 2.4-fold higher in HL-60 compared to 5637 cells. This data is in excellent agreement with the enhanced cytotoxicity of rViscumin toward HL-60 cells (see Figure 5).

Generating CHO-K1 cell susceptibility toward rViscumin by exogenous gangliosides

Gangliosides added exogenously to culture medium are taken up by a wide range of cells *in vitro* (Saqr *et al.*, 1993) and incorporated into the plasma membrane by a time- and concentration-dependent process (Radsak *et al.*, 1982). CHO-K1 cells have been found to express predominantly GM3 (Figure 6A, lane b). Other gangliosides are either absent or present only in trace quantities. Attempts were made to render CHO-K1 cells susceptible toward rViscumin. For that purpose, increasing amounts of HGG harboring the rViscumin receptor IV^{6}nLc4Cer as the prevalent ganglioside were applied to CHO-K1 cell cultures and allowed to incorporate. As shown in Figure 7, incubation of CHO-K1 cells with increasing concentrations of HGG prior to rViscumin treatment reduced the cell viability in a concentration-dependent manner. Cells prewashed with serum-free medium before rViscumin application revealed viabilities decreasing from 85.7% down to 31.5% (relative to control cultures without gangliosides and rViscumin) after incubation with 25 µM up to 200 µM HGG, respectively. Prewashing with serum-supplemented medium enhanced the cytotoxic effect leading to somewhat diminished viabilities at the respective HGG concentrations. Gangliosides alone had no effect on CHO-K1 cells up to a concentration of 100 µM; after treatment with 200 µM HGG viability was slightly decreased by about 12% compared to control cultures without gangliosides. From this data it was concluded that insertion of the receptor ganglioside IV^{6}nLc4Cer from HGG, which represents the major ganglioside with Neu5Acα2-6Galβ1-4GlcNAc terminus in

Table IV. Quantification of rViscumin receptors IV^{6}nLc4Cer (C24-fatty acid) and IV^{6}nLc4Cer (C16-fatty acid) in ganglioside fractions of 5637 and HL-60 cells

Cell type	IV^{6}nLc4 (C24-fatty acid) = compound 1*	IV^{6}nLc4 (C16-fatty acid) = compound 2*	IV^{6}nLc4 (C24- + C16-fatty acid) = compound 1 + compound 2
5637 cells			
ng/10^7 cells	33.1	7.9	41
mol/10^7 cells	2.036×10^{-11}	5.218×10^{-12}	2.558×10^{-11}
Molecules/cell	1.23×10^6	3.14×10^5	1.54×10^6
HL-60 cells			
ng/10^7 cells	84.9	13.5	98.4
mol/10^7 cells	5.221×10^{-11}	8.885×10^{-12}	6.109×10^{-11}
Molecules/cell	3.14×10^6	5.35×10^5	3.68×10^6

Performed by TLC immunostaining with anti-IV^{6}nLc4Cer antibody.
*See Figure 6.

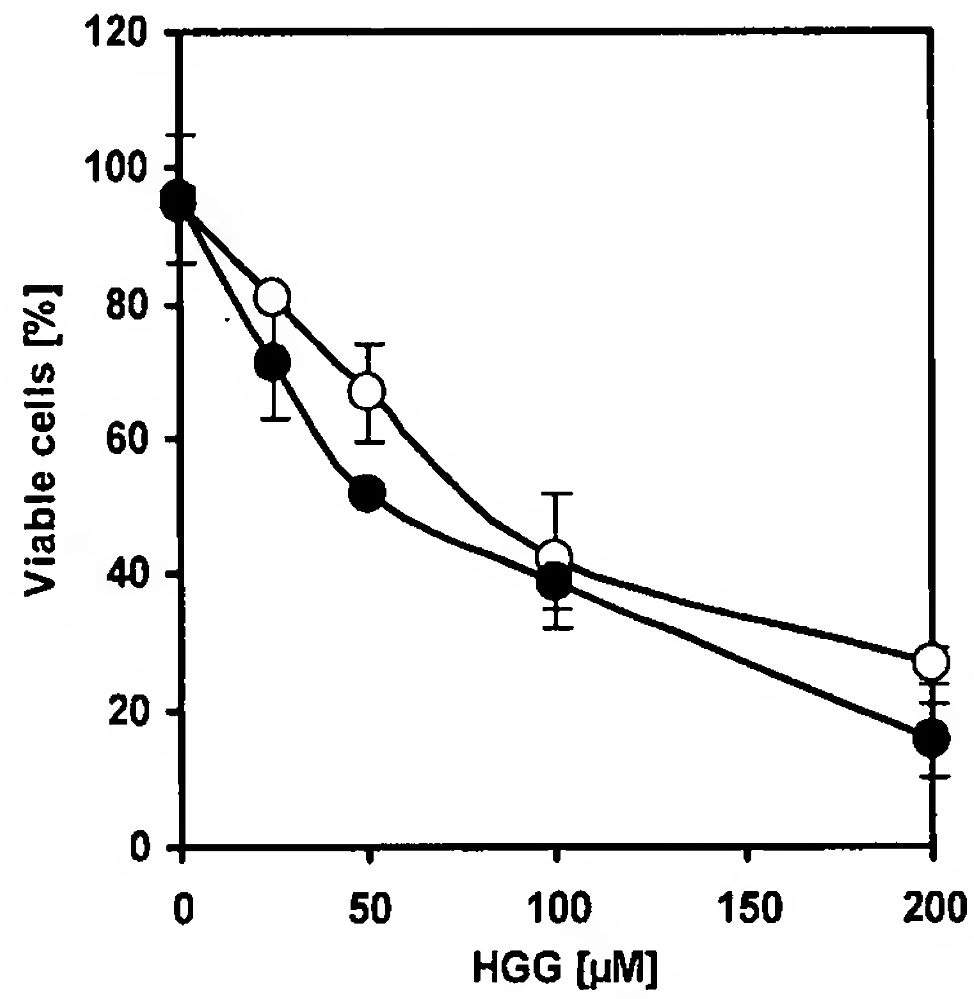

Fig. 7. Generating rViscumin sensitivity toward CHO-K1 cells by incorporation of exogenous gangliosides. Viability of cell layers, washed either with serum-free (open circles) or serum-containing medium (closed circles), was plotted versus HGG concentrations. Increasing amounts of HGG were applied to microwell cell cultures for 48 h. After washing, the cell layers were incubated with 5.26 nM rViscumin for 48 h. Viability was measured in the WST-1 cytotoxicity assay and calculated as percentage relative to control cultures without gangliosides and rViscumin.

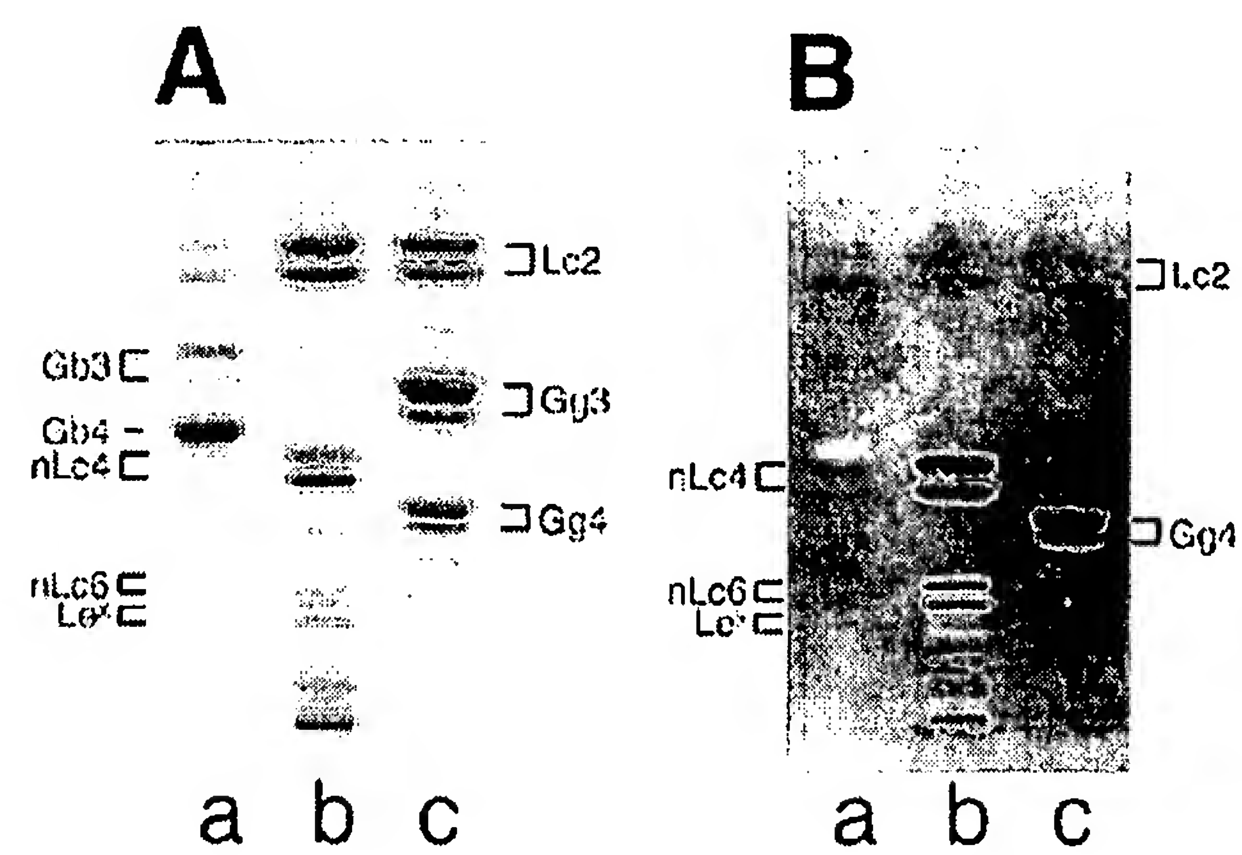

Fig. 8. Orcinol stain (A) and ricin TLC overlay assay (B) with neutral GSLs. Lanes a: 10 µg of neutral GSLs from human erythrocytes; lanes b: 15 µg of neutral GSLs from human granulocytes; lanes c: 20 µg of neutral GSLs from MDAY-D2 cells. For structures and graded appearance on the TLC immunostain see Table I.

the mixture, turned CHO-K1 cells from rViscumin-resistant to -sensitive cells, thereby confirming the biological function of IV^{6}nLc4Cer as a true and physiologically relevant rViscumin receptor.

TLC overlay assay of ricin with neutral GSLs and gangliosides

Ricin (*Ricinus communis* lectin) is well accepted to represent a type II ribosome-inactivating protein with galactose specificity. This was tested with the same set of GSL references used for exploring rViscumin binding specificity. The orcinol stain (Figure 8A) and the parallel TLC-binding assay of ricin with neutral GSLs disclosed a weak staining of LacCer (Galβ1-4Glcβ1-1Cer) in all three reference mixtures (Figure 8B). The N-acetyl group of the penultimate GlcNAc obviously increased the strength of binding to nLc4Cer and nLc6Cer (both with Galβ1-4GlcNAc terminus) as shown in lane b of Figure 8B (Table I). Fucosylation of the nLc6Cer-core completely abolished ricin binding, indicated by failed recognition of Lewis^x-GSL. Significant but less intensive ricin adhesion compared to, for example, nLc4Cer, was observed for Gg4Cer bearing the Galβ1-3GalNAc-disaccharide at the nonreducing end of the oligosaccharide sequence. Gb4Cer (GalNAcβ1-3Gal terminus) was negative and Gb3Cer (Galα1-4Gal terminus) exhibited weak signals. The order of binding preference of ricin toward neutral GSLs, deduced from TLC immunostainings, were the sequences Galβ1-4GlcNAc > Galβ1-3GalNAc > Galβ1-4Glc > Galα1-4Gal.

Finally, TLC ricin overlay assays performed with gangliosides gave negative results for all terminally sialylated ganglio- and neolacto-series gangliosides (Figure 9B). A faint positive reaction was observed for GM1, a Gg4Cer-core ganglioside with Neu5Ac bound to the internal position II of the tetraosyl-backbone. Sialylation at position IV of Gg4Cer (GD1a) and the disialo group Neu5Acα2-8Neu5Acα2-3R at position II of

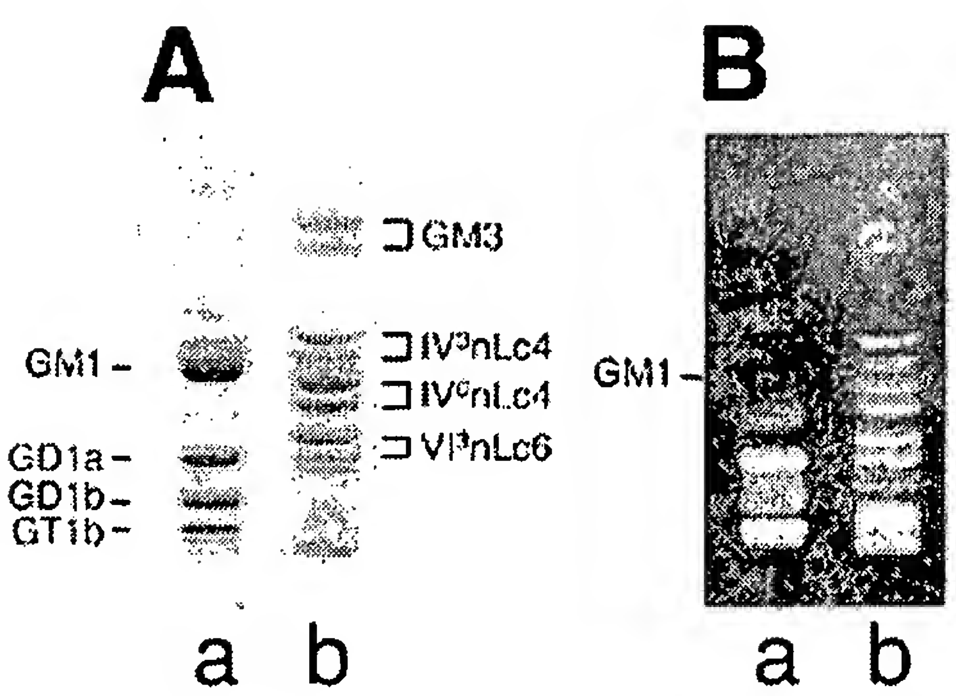

Fig. 9. Orcinol stain (A) and ricin TLC overlay assay (B) with gangliosides. Lanes a: 10 µg human brain gangliosides; lanes b: 8 µg HGGs. For structures and graded appearance on the TLC immunostain see Table II.

GD1b, completely prevented ricin binding. Most important with regard to rViscumin specificity, no binding to any neolacto-series gangliosides could be observed as indicated by unstained white bands in the intentionally overstained background of the TLC immunoassay plate (Figure 9B). According to the data of these TLC immunostaining investigations, ricin can be unequivocally considered as a galactose-binding lectin, clearly distinguishable from rViscumin, which was shown to represent a sialic acid–specific lectin.

Discussion

The first step in the biological action of viscumin (ML-I) is recognition and binding of specific ligands on the surface of target cells. When glycoproteins with a complex pattern of glycosylation serve as source for the presentation of glycoligands, the individual contribution of each epitope is difficult to discern. Consequently, a huge number of binding and inhibition assays with chemically well-defined model ligands have been performed to better understand the initial process of ML-I adhesion. For that purpose, carbohydrate binding specificity of the galactose-binding ML-I was studied by competetive inhibition with monosaccharides, monosaccharide derivatives, disaccharides, and compounds containing multiple galactosyl terminals (Lee *et al.*, 1992; Möckel *et al.*, 1997). It was found that ML-I has a broad range of affinity for Galα- and Galβ-linked sequences, revealing primarily recognition of the terminal galactose unit irrespective of the anomeric linkage (Lee *et al.*, 1994; Gupta *et al.*, 1996).

Although the conformational parameters of derivatized neutral disaccharide ligands before and after complex formation with the galactose-binding *Viscum album* lectin have been studied in detail (Gilleron *et al.*, 1998), little is known about the interaction of ML-I with naturally occuring sialooligosaccharides and sialoglycoproteins. The precipitability of human α1-acid glycoprotein and fetuin with ML-I was considerably decreased after desialylation (Wu *et al.*, 1995), but on the contrary the poor reactivity of rat sublingual sialoglycoprotein with ML-I increased substantially after removal of sialic acid. The inhibition of the glycoprotein-lectin interaction by Neu5Acα2-3/α2-6Galβ1-4Glc was taken as evidence that ML-I is specific for sialic acid. Substitution of the N-acetyllactosamine sequences of oligosaccharides and glycopeptides by sialic acid residues either at O-3 or O-6 of galactose was found to slightly enhance the affinity of ML-I (Debray *et al.*, 1994).

Following the approach with chemically well-defined model ligands (Lee *et al.*, 1992), a panel of strictly defined neoglycoconjugates and synthetic oligosaccharides were systematically tested to determine their individual binding and inhibitory potency (Galanina *et al.*, 1997). Among the two natural isomers of sialyllactose, the α2-6 form displayed a higher level of inhibitory capacity than the α2-3 derivative. However, from all these highly sophisticated binding and inhibition assays performed so far, the natural ligands on immunomodulatory potent and/or tumor target cells being responsible for B-chain-specific binding of the ML remained yet unidentified and rather obscure.

Among all the gangliosides tested in this study, terminally sialylated neolacto-series gangliosides with a Neu5Acα2-6Galβ1-4GlcNAc epitope at the nonreducing terminus, which have been isolated from human granulocytes, were the exclusive targets for rViscumin-mediated adhesion. The isomeric gangliosides with Neu5Acα2-3Galβ1-4GlcNAc sequences and neutral GSLs with Galβ1-4GlcNAc epitope were devoid of binding activity. LacCer (Galβ1-4Glcβ1-1Cer) was found to be the only, but rather slightly interacting neutral GSL with marginal receptor potency in comparison to α2-6-sialylated gangliosides. However, α2-6-sialylated lacto-series gangliosides with Galβ1-3GlcNAc-core cannot be excluded as possible rViscumin ligands at this stage of research. This aspect of further receptor characterization is a part of our future investigations.

Cancer cells are often characterized by the presence of "tumor-associated" GSL-antigens, and major progress has been made over the past two decades on structural identification of these antigens (Hakomori, 1998). None of these structures are truly tumor-specific, and many GSLs to which antitumor antibodies responses are directed are also found in normal tissues. However, a large number of anti-GSL antibodies showed specific or preferential reactivity with tumor cells and no reactivity with normal cells or tissues, based on organizational differences of membrane GSLs in tumor cells versus normal cells. To elucidate the potential role of terminally α2-6-sialylated gangliosides as tumor-associated antigens, careful (re)investigations of rViscumin-susceptible tumor cell lines and/or tumor tissues has to be performed with regard to their specific expression of neolacto-series gangliosides with Neu5Acα2-6Galβ1-4GlcNAc-epitopes.

Following cell surface binding of *V. album* lectin and ensuing signaling, several cellular responses (such as secretion of cytokines, namely, tumor necrosis factor-α, interleukin-1, and interleukin-6 by human peripheral blood mononuclear cells) can be measured in the nontoxic dose range *in vitro* and *in vivo* (Hajto *et al.*, 1990; Gabius *et al.*, 1992; Möckel *et al.*, 1997; Stein *et al.*, 1998; Ribéreau-Gayon *et al.*, 1997). Specific immune responses against tumor-associated antigens mediated by T lymphocytes and nonspecific immune responses induced by cells of the mononuclear phagocyte system and natural killer cells appear to participate in naturally acquired resistance against neoplastic diseases. Studies on animals and humans proved that ML-I causes significant increase and activation of natural killer cells and enhances phagocytotic activity of granulocytes and monocytes (Hajto *et al.*, 1989, 1990). T cell activation and preferential expansion of $CD8^+$ T cells mediating

the cytotoxic effect has been reported as well (Baxevanis *et al.*, 1998). The question currently remains unanswered of which type of oligosaccharides, that is, protein- or lipid-bound, is recognized by ML-I and may be responsible for eliciting the enumerated immunoresponses. Concerning the sialic acid specificity of MLB, amino acid sequencing (Huguet Soler *et al.*, 1998) and molecular cloning of the B-chain in *E. coli* (Eck *et al.*, 1999b) led to the speculation that rViscumin possesses at least three binding sites, as recently described for ricin (Frankel *et al.*, 1996; Steeves *et al.*, 1999).

Finally, emphasizing the functional importance of receptor clustering described for neoglycoconjugates (André *et al.*, 1997), GSLs and particularly gangliosides are ideal candidates to fulfill the requirements for being effective rVscumin receptors. We presented a body of data that α2-6-sialylated gangliosides might be involved in B-chain-dependent signal triggering, thereby truly resembling cellular targets for rViscumin acting as B-chain-mediated immunomodulator and cytotoxic agent.

Materials and methods

Mass production of cells

CHO-K1 cells (ATCC CCL-81) and HL-60 cells (promyelotic human leukemia cell line, ATCC CCL-240) were cultivated with an 1:1 mixture of Dulbecco's modified Eagle medium (DMEM) and Ham's F12 medium and the human bladder carcinoma cell line 5637 (ATCC, HTB-9) in RPMI 1640. The cells were routinely passaged in conventional culture flasks (Nunc, Wiesbaden, Germany) at 37°C in a humidified 5% (v/v) CO_2 air-atmosphere. The media were buffered with 2.1 g/L $NaHCO_3$ and supplemented with 10% fetal calf serum (v/v). Cells were grown in the presence of 50 mg/L gentamycin and 2.5 mg/L amphotericin B.

Cell production of 5637 cells was performed in 175-cm^2 culture flasks. HL-60 and CHO-K1 cells were produced in a 1-L SuperSpinner (B. Braun Biotech International, Melsungen, Germany) equipped with a membrane stirrer for optimized oxygen supply of the cells (Heidemann *et al.*, 1994). CHO-K1 cells were propagated on Cytodex-1 microcarriers (Amersham Pharmacia Biotech, Uppsala, Sweden) as described in detail by Duvar *et al.* (1996) and Müthing *et al.* (1996b). The physiological set points for spinner cultivations were: 37°C; pH 7.2; aeration with air; stirrer 40 rpm. After reaching the final cell densities, the cells were harvested and washed twice with phosphate buffered saline (PBS) before chloroform/methanol extraction.

Cytotoxicity assay

Cell viability and *in vitro* cytotoxicity were assayed using 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1, Roche Diagnostics, Mannheim, Germany). Cytotoxicity of rViscumin was analysed with HL-60, 5637, and CHO-K1 cells. Medium for HL-60 cells was serum-free X-Vivo 15 (Biowhittaker, Walkersville, MO), RPMI 1640 with 2.5% fetal calf serum for 5637, and DMEM/F12 with 1% fetal calf serum for CHO-K1 cells. Indirect measurement of cell viability was achieved by conversion of the soluble sulfonated tetrazolium salt into formazan by cells according to Ishiyama *et al.* (1993). Samples were tested in six replicates, and the absorbance was determined at 450 nm for each well.

To assay cytotoxicity, 8×10^3 5637, 1×10^4 CHO-K1, or 1.8×10^4 HL-60 cells, respectively, were seeded per well in 96-well tissue culture plates (Nunc) and incubated with rViscumin in a final volume of 100 µl for 72 h at 37°C. For treatment, an adequate concentration range of rViscumin was chosen according to the sensitivity of the respective cell line (see Figure 5). After 72 h 10 µl WST-1 solution were added to the cell cultures and incubated for 3–4 h at 37°C. The amount of dye, which is proportional to the number of viable cells, was measured in a microwell plate reader (THERMOmax, Molecular Devices, Munich, Germany).

rViscumin, ricin, and anti-lectin antibodies

The heterodimeric recombinant ML (rML) rViscumin, composed of rMLA (A-chain) and rMLB (B-chain) (Eck *et al.*, 1999b), was used for analyzing binding specificity toward neutral GSLs and gangliosides in microwell adsorption and TLC overlay binding assay. GSL-bound rViscumin was detected with the mouse IgG monoclonal anti-rMLA antibody TA5 (Tonevitsky *et al.*, 1995).

R. communis lectin (ricin) was a kind gift of Dr. F. Stirpe (University of Bologna, Italy). Polyclonal rabbit anti-ricin antibody was purchased from Sigma-Aldrich Chemie GmbH (Traunstein, Germany; catalog no. R-1254).

rViscumin and ricin are highly potent toxins when reaching the body via the parenteral route (intravenous, subcutaneous, and intramuscular), whereas the enteral route (oral) toxicity is relatively low. Thus, handling of both substances should be done with utmost care, using gloves and avoiding highly concentrated working solutions and sharp or pointed tools.

Generation of rViscumin-susceptible CHO-K1 cells by exogenous gangliosides

The rViscumin-resistant CHO-K1 cells were tested for change in sensitivity to rViscumin after exogenous application of gangliosides in cell cultures. Cells were seeded at a density of 5×10^3 cells per well into a 96-well plate in serum-free CHO-1 medium (Biowhittaker, MD) and cultured for 24 h. The medium was then withdrawn and replaced by CHO-K1 medium containing 0 (control), 25, 50, 100, and 200 µM, respectively, of a preparation of total HGG. Gangliosides were allowed to incorporate into cells for 48 h under culture conditions at 37°C. After this incubation the cells were extensively washed either with serum-free or 5% fetal calf serum–containing CHO-K1 medium, followed by a 48-h treatment with 5.26 nM rViscumin. Viability was examined by WST-1 staining as described in comparison to untreated control cultures (without gangliosides and rViscumin). All cell culture assays were performed in triplicate in 100 µl volumes per well.

Isolation of GSLs from in vitro cultivated cells

GSLs from *in vitro* propagated cells were isolated according to standard procedures (Ledeen and Yu, 1982) and as described in detail by Duvar *et al.* (1997). Gangliosides were isolated by anion exchange chromatography on DEAE-Sepharose CL-6B (Pharmacia Fine Chemicals, Freiburg, Germany) as reported by Müthing *et al.* (1987). The ganglioside fractions were incubated for 1 h at 37°C in aqueous 1 N NaOH to saponify phospholipids followed by neutralization with acetic acid and dialysis. Gangliosides were further purified by adsorption

chromatography on Iatrobeads 6RS-8060 (Macherey-Nagel, Düren, Germany) as described by Ueno *et al.* (1978).

Reference neutral GSLs and gangliosides

Neutral GSLs from human erythrocytes (globo-series), human granulocytes (neolacto-series), and murine MDAY-D2 cells (ganglio-series) served as references for microwell adsorption assays and TLC overlay binding assays. Neutral GSL fractions contained (in the order of the relative amounts in each fraction starting with the dominant compound) Gb4Cer, Gb3Cer, and LacCer (erythrocytes); LacCer, nLc4Cer, nLc6Cer, and Le^x-GSL (granulocytes; Müthing *et al.*, 1994; Müthing and Kemminer, 1996); and Gg4Cer, Gg3Cer, and LacCer (MDAY-D2; Schwartz *et al.*, 1985; Müthing and Cacic, 1997).

A ganglioside mixture containing GM3, IV^{3}nLc4Cer, IV^{6}nLc4Cer, and VI^{3}nLc6Cer as the major constituents was isolated from human granulocytes (total HGGs) as previously described (Müthing *et al.*, 1993, 1996a).

A preparation of human brain gangliosides, composed of GM1, GD1a, GD1b, and GT1b, was purchased from Supelco (Bellefonte, PA).

Anion-exchange HPLC separation of terminally α2-3- and α2-6-sialylated neolacto-series gangliosides

For the separation of α2-3- and α2-6-sialylated monosialogangliosides HPLC was carried out with the Superformance universal glass-cartridge device of Merck, earlier published in detail (Müthing and Unland, 1994; Müthing, 2000). A glass cartridge (150 mm × 10 mm) filled with Fractogel EMD TMAE-650(S) (Merck, No. 20286) was loaded with total gangliosides from human granulocytes (= HGG, see previous methods). TMAE-Fractogel-bound gangliosides were eluted with a linear ammonium acetate gradient, pooled, and desalted. Four fractions were obtained, comprising terminally α2-3 sialylated (HGG1 and HGG2) and terminally α2-6-sialylated neolacto-type monosiaolgangliosides (HGG3 and HGG4). The fractions HGG1 and HGG2 contained IV^{3}nLc4Cer and VI^{3}nLc6Cer, respectively. IV^{6}nLc4Cer represents the only compound in fraction HGG3. HGG4 contained IV^{6}nLc4Cer and VI^{6}nLc6Cer plus minor quantities of VIII^{6}nLc8Cer. All gangliosides separate as double bands on thin-layer chromatograms due to substitution of the sphingosine moiety with C24-(upper band) and C16-fatty acid (lower band). The structures of IV^{3}nLc4Cer, IV^{6}nLc4Cer, and VI^{3}nLc6Cer have been determined by fast atom bombardment–MS and methylation analysis, as described elsewhere (Müthing *et al.*, 1993). The terminally α2-3-sialylated gangliosides IV^{3}nLc4Cer and VI^{3}nLc6Cer have been reinvestigated by electrospray ionization–quadrupole time-of-flight MS (ESI-QTOF-MS) as recently reported (Metelmann *et al.*, 2000). Terminally α2–6-sialylated gangliosides VI^{6}nLc6Cer and VIII^{6}nLc8Cer were structurally characterized in this study by ESI-QTOF-MS (see the following section).

ESI-QTOF-MS

Gangliosides of HPLC-purified fractions HGG3 and HGG4 were analyzed by nanoESI-MS and MS/MS using a QTOF mass spectrometer (Micromass, Manchester, UK) equipped with a nanospray manipulator. Negative ion mode was used exclusively for the ganglioside analysis. After selecting the precursor ion of interest with the first quadrupole, collision-induced dissociation was applied to obtain fragment ions for MS/MS sequencing (for details see Metelmann *et al.*, 2001). The nomenclature introduced by Domon and Costello (1988) was used for assignment of fragment ions.

Polyclonal anti-Neu5Acα2-6Galβ1-4GlcNAc-R antibody

At the age of 12 weeks a chicken was immunized according to the method of Kasai *et al.* (1980). HPLC-purified IV^{6}nLc4Cer was prepared from human granulocytes as described. One milligram of the ganglioside was adsorbed to 1 mg permethylated bovine serum albumin (Serva, Heidelberg, Germany) in PBS. The solution was emulsified with an equal part of Freund's adjuvant (Difco, Detroit, MI) in a final volume of 1 ml and administered at multiple intramuscular sites. Preimmune serum was taken just before immunization. After 4 weeks, the chicken was boostered and exsanguinated 14 days later.

High-performance thin-layer chromatography

GSLs were separated on high-performance thin-layer chromatography plates (HPTLC plates, size 10 cm × 10 cm, thickness 0.2 mm, Merck; Art. No. 5633). Neutral GSLs were chromatographed in solvent I (chloroform/methanol/water, 120/70/17, each by volume) and gangliosides in solvent II (chloroform/methanol/water, 120/85/20, each by volume), the latter supplemented with 2 mM $CaCl_2$. Neutral GSLs were visualized with orcinol (Svennerholm, 1956) and gangliosides with orcinol or resorcinol (Svennerholm, 1957). IV^{6}nLc4Cer-gangliosides of the HGG fraction were quantified by densitometry with a CD60 scanner (Desaga, Heidelberg, Germany) equipped with an IBM-compatible personal computer and densitometric software. Chromatographed bands were measured in reflectance mode at 580 nm (resorcinol) with a light beam slit of 0.1 × 2 mm.

Neutral GSLs from human granulocytes and MDAY-D2 cells as well as gangliosides from human granulocytes appear as double bands on TLCs as already described.

TLC overlay assay

Secondary rabbit anti-chicken IgY, goat anti-mouse IgG and IgM, and goat anti-rabbit IgG antisera, all affinity chromatography-purified and labeled with alkaline phosphatase, were purchased from Dianova (Hamburg, Germany) and used in a 1:2000 dilution (Duvar *et al.*, 1997). The TLC immunostaining procedure was carried out according to Magnani *et al.* (1982) with some modifications. After TLC of GSLs the silica gel was fixed with polyisobutylmethacrylate (Plexigum P28, Röhm, Darmstadt, Germany) as described by Müthing and Mühlradt (1988). Two reviews concerning the details of the TLC immunostaining procedure have been published (Müthing, 1996, 1998).

Terminally α2-6-sialylated neolacto-series gangliosides were detected by the chicken polyclonal anti-Neu5Acα2-6Galβ1-4GlcNAc-R antibody. The HPTLC plate was soaked for 15 min with 1% (w/v) bovine serum albumin in PBS (solution A) and then overlayed for 1 h with the primary antibody diluted 1:1000 in solution A. The plate was washed three times with 0.05% (v/v) Tween 21 in PBS (solution B) and incubated for 1 h with alkaline phosphatase-labeled rabbit anti-chicken IgY antibody diluted in solution A. The plate was then washed three times with solvent B and once with glycine buffer (0.1 M glycine, 1 mM $ZnCl_2$, 1 mM $MgCl_2$, pH 10.4) to remove phosphate. Bound antibodies were visualized by color development of blue indigo–like stable stain after incubation

of the plate with 0.05% (w/v) 5-bromo-4-chloro-3-indolyl-phosphate (Biomol, Hamburg, Germany) in glycine buffer.

The rViscumin binding activity toward GSLs was detected after prewashing the plate with 0.1 g/L Tween 80 in PBS (PBS-T80), followed by overlaying the chromatogram for 1 h with 1 μg/ml rViscumin diluted in PBS-T80. The plate was washed with solution B, incubated for 15 min with solution A, and then overlayed with the murine anti-rMLA monoclonal antibody TA5 (1 μg/ml in solution A). The next steps were performed as already described. Secondary alkaline phosphatase labeled anti-mouse IgG and IgM was used for the detection of bound mouse TA5 antibody.

The ricin overlay binding assay was performed according to the protocol described for rViscumin. Ricin was used in a working concentration of 1 μg/ml in PBS-T80; the polyclonal rabbit anti-ricin antibody was employed in 1:200 dilution and detected with alkaline phosphatase labeled secondary anti-rabbit antibody.

Determination of cellular quantities of IV^{6}nLc4Cer

The amounts of IV^{6}nLc4Cer expressed by HL-60 and 5637 cells were quantified by scanning of TLC bands stained with the anti-IV^{6}nLc4Cer antibody. Defined quantities of HGG reference IV^{6}nLc4Cer ganglioside (calibration curve) were scanned in comparison to ganglioside fractions of defined cell numbers ranging from 1×10^6 to 2×10^7 cells. The blue-colored immuno-stained double bands were scanned in reflectance mode at 630 nm. The numbers of IV^{6}nLc4Cer molecules per cell were calculated by use of Avogadro's constant (6.02252×10^{23} molecules per mol) and the known molecular weight of 1626 for IV^{6}nLc4Cer substituted with C24-fatty acid (upper band, compound 1) and the molecular weight of 1516 for IV^{6}nLc4Cer substituted with C16-fatty acid (lower band, compound 2).

Microwell adsorption assay

The microwell adsorption assays were carried out with neutral GSLs and gangliosides on polystyrene microtiter plates (MaxiSorp F96 immuno plates; Nunc) at room temperature. Microwells were loaded with GSLs in 100 μl methanol, starting with a defined GSL amount followed by three serial 1:2 dilutions. Neutral GSLs from human erythrocytes: 1.25, 2.5, 5, and 10 μg; neutral GSLs from human granulocytes: 1.88, 3.75, 7.5, and 15 μg; neutral GSLs from MDAY-D2: 2.5, 5, 10, and 20 μg; human granulocyte gangliosides and human brain gangliosides: 1.25, 2.5, 5, and 10 μg. Methanol was evaporated by exposure of the plate in a dry atmosphere for 45 min at 37°C. The solutions used were the same as described for the TLC overlay binding assay.

All incubation steps were performed in volumes of 100 μl per well. The GSL-coated microwells were incubated for 15 min with PBS-T80 and then provided with 1 μg of rViscumin dissolved in PBS-T80 (0.01 μg/μl). After 1 h incubation the wells were washed three times with solvent B, soaked for 15 min in solution A, and then loaded for 1 h with the murine anti-rMLA monoclonal antibody TA5 (1 μg/ml in solution A). The wells were washed three times with solvent B and incubated with alkaline phosphatase-labeled anti-mouse IgG and IgM secondary antibody diluted 1:2000 in solution A. After 1-h incubation and threefold washing with solution B, bound antibodies were visualized with di-sodium-4-nitro-phenylphosphatehexahydrate (16 mM in 0.1 M glycine buffer pH 10.4). Enzyme activity was recorded after 20 min at 405 nm with an ELISA microplate autoreader (EL311, Bio-Tec Instruments, Winooski, VT).

Acknowledgments

We are grateful to Prof. Dr.-Ing. J. Lehmann, head of the Institute of Cell Culture Technology (University of Bielefeld) for his generous support. We thank Mrs. U. Baillie for critical reading and correction of the manuscript and Dr. H. Ziehr (GBF, Braunschweig) for valuable advice. The help for preparing figures by Dr. I. Meisen and P. Bulau is also gratefully acknowledged.

Abbreviations

CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle medium; ESI-QTOF, electrospray ionization–quadrupole time-of-flight; GSL, glycosphingolipid; HGG, human granulocyte gangliosides; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; ML, mistletoe lectin; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PBS, phosphate buffered saline; TLC, thin-layer chromatography; WST-1, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate.

The designation of the gangliosides and their core structures follow the IUPAC-IUB recommendations (1998) and the nomenclature of Svennerholm (1963). Structures of neutral GSLs and gangliosides are listed in Table I and II, respectively.

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